

Environmental factors influence virulence of
Pseudomonas aeruginosa

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Contents

1	INTRODUCTION.....	11
1.1	<i>Pseudomonas aeruginosa</i> – an opportunistic pathogen	12
1.2	Chronic <i>P. aeruginosa</i> infections.....	12
1.3	Biofilm development	13
1.4	Resistance of biofilms	14
1.5	Factors contributing to biofilm formation.....	15
1.5.1	Motility and attachment organelles.....	15
1.5.2	Exopolysaccharides	16
1.5.3	Rhamnolipids	18
1.6	Intercellular signaling (quorum sensing).....	18
1.7	Siderophores.....	19
1.8	Two component systems.....	20
1.8.1	Phosphate (PHO) regulon.....	21
1.9	C-di-GMP	23
1.9.1	Downstream targets of c-di-GMP	24
1.9.2	Impact of c-di-GMP on the virulence phenotype	25
1.10	Type three secretion system of <i>P. aeruginosa</i>	26
1.11	Aim of the work	28
2	MATERIALS AND METHODS.....	29
2.1	Bacterial strains.....	30
2.1.1	<i>Pseudomonas aeruginosa</i>	30
2.1.2	<i>Escherichia coli</i>	30
2.2	Culture media.....	30
2.2.1	Luria Bertani (LB) medium (Sambrook <i>et al.</i> , 1989).....	30
2.2.2	Columbia blood agar plates (Becton Dickinson)	31

I. CONTENTS

2.2.3	DeMoss minimal medium (Frank and Demoss, 1959)	31
2.2.4	Tryptone soymeal broth (TSB)	31
2.3	Buffers and solutions	32
2.3.1	1x PBS (Phosphate-buffered saline)	32
2.3.2	Agarose Electrophoresis buffers	32
2.3.3	SDS-PAGE buffers	32
2.3.4	Western blot buffers	33
2.4	Plasmids and constructs	34
2.5	Primers	34
2.6	Preparation of permanent cultures	35
2.7	Static quantitative biofilm assay	35
2.8	Biofilm assay and 3D-imaging by confocal scanning laser microscope ...	36
2.9	Congo red binding assay	36
2.10	Swimming motility assay	37
2.11	Flow cytometric analysis of ExoT expression	37
2.12	Pyoverdine and pyochelin measurement	38
2.13	Nucleic acids techniques	38
2.13.1	Isolation of genomic DNA	38
2.13.2	Isolation of plasmid DNA	38
2.13.3	Agarose gel electrophoresis	39
2.13.4	Cloning of the PCR products	39
2.13.5	RNA isolation	45
2.13.6	cDNA synthesis	45
2.13.7	Semi-quantitative RT-PCR	46
2.13.8	Quantitative real time RT- PCR	48
2.13.9	Transcriptome analysis	49
2.14	Protein techniques	51
2.14.1	SDS polyacrylamide gel electrophoresis (SDS-PAGE)	51

I. CONTENTS

2.14.2	Colloidal Coomassie blue staining	52
2.14.3	Western blot.....	52
2.14.4	Overexpression of protein in <i>E.coli</i>	54
3	RESULTS.....	56
3.1	The Pho regulon negatively influences biofilm formation in <i>P. aeruginosa</i>	57
3.2	Bioinformatic-based search for candidate Pho regulated biofilm genes ...	60
3.3	PA3258 is a member of Pho regulon but not PA1727 or PA2567	61
3.4	PA3258 contributes to the inhibition of biofilm formation.....	63
3.5	Pho regulon influences the biofilm structure of <i>P. aeruginosa</i>	66
3.6	Expression of CupA fimbriae do not contribute to the Pho- dependent biofilm phenotype	67
3.7	The Psl polysaccharide does not contribute to the Pho- dependent biofilm phenotype	67
3.8	Expression and purification of the RapA protein to assess the phosphodiesterase activity of RapA <i>in vitro</i>	68
3.9	The Pho regulon influences the type three secretion system	69
3.10	A PA14 <i>flgF</i> mutant negatively regulates biofilm formation similar to the Pho regulon.....	71
3.11	TTSS expression is negatively influenced by the FlgF protein.....	72
3.12	Transcriptome analysis of the <i>flgF</i> mutant.....	73
3.13	The Pho regulon influences pyochelin and pyoverdine production	79
3.14	Pyochelin and pyoverdine do not affect swimming motility or TTSS	80
4	DISCUSSION	82
4.1	The Pho regulon influences biofilm formation and TTSS	83
4.2	Expression of flagella influences biofilm formation and TTSS.....	86
4.3	Transcriptome analysis does not uncover cooperative regulation of biofilm formation and TTSS expression.....	87
4.4	PHO regulon and Flagella expression influence pyochelin and pyoverdine production	88

I. CONTENTS

4.5	Conclusion	91
5	SUMMARY	92
6	ZUSAMMENFASSUNG.....	95
7	REFERENCES	98

List of Figures

Figure (1) Stages of the biofilm development.	14
Figure (2) Schematic diagram of the flagellar structure in <i>P. aeruginosa</i>	15
Figure (3) Synthesis and degradation of c-di-GMP.	24
Figure (4) Schematic structure of the type three secretion injectosome in <i>P. aeruginosa</i>	27
Figure (5) Restriction map of the shuttle vector pUCP20.	41
Figure (6) Restriction map of the expression vector pET21a(+) (Novagen).	42
Figure (7) Assembly of the western blot.	53
The SDS-PAGE gel and the PVDF-membrane were carefully put together in between 2 layers of 3 Whatman-paper. The gel was directed towards the cathode plate of the Trans-Blot SD transfer system while the PVDF-membrane was directed towards the anode.	53
Figure (8) Biofilm formation in PA14 wild-type, <i>phoB</i> mutant, <i>phoB</i> mutant complemented with pUCP20: <i>phoBR</i> and <i>pstS</i> mutant.	57
Figure (9) Quantification of biofilm formation in the PA14 strain (A) and the PAO1 strain background (B).	59
Figure (10) Domain organisation of the PA3258, PA2567 and PA1727 proteins.	60
Figure (11) Semi-quantitative RT-PCR for transcription of PA1580 (a), PA3258 (b), PA2567 (c) and PA1727 (d) in <i>P. aeruginosa</i> PA14 wild-type under low and high phosphate and in the <i>phoB</i> and the <i>pstS</i> mutant under low phosphate medium conditions.	62
Figure (12) Quantification of biofilm formation in the PA14 strain (A) and the PAO1 strain background (B).	65
Figure (13) Biofilm structures (3D visualization) of PA14 wild-type, <i>phoB</i> mutant, <i>phoB</i> mutant complemented with pUCP20: <i>phoBR</i> , <i>rapA</i> mutant, <i>rapA</i> mutant complemented with pUCP20: <i>rapA</i> and <i>pstS</i> mutant.	66
Figure (14) Western blot of CupA fimbriae expression in <i>P. aeruginosa</i>	67
Figure (15) Congo red binding assay of <i>P. aeruginosa</i> PA14 <i>phoB</i> , <i>rapA</i> mutants and wild-type control.	68
Figure (16) Analytical SDS-PAGE showing the overexpression of the RapA protein in <i>E. coli</i> BL21 pET-21a(+) <i>rapA</i>	69

II. LIST OF FIGURES

Figure (17) pExoT-gfp expression in <i>P. aeruginosa</i> PA14 as measured by FACS analysis.....	70
Figure (18) Biofilm formation of the <i>P. aeruginosa</i> PA14 wild-type and <i>flgF</i> mutant.	71
Figure (19) Swimming motility of <i>P. aeruginosa</i> PA14 wild-type, <i>flgF</i> mutant, <i>flgF</i> mutant complemented with pUCP20: <i>flgF</i>	72
Figure (20) pExoT-gfp expression in the <i>P. aeruginosa</i> PA14 wild-type, <i>flgF</i> mutant and the mutant complemented with pUCP20: <i>flgF</i> as measured by FACS analysis..	73
Figure (21) Swimming of <i>P. aeruginosa</i> PA14 wild-type control, <i>phoB</i> mutant, <i>rapA</i> mutant.....	74
Figure (22) Pyochelin production by <i>P. aeruginosa</i> PA14 wild-type control and the <i>phoB</i> mutant under low and high phosphate conditions.	79
Figure (23) Pyoverdine production by <i>P. aeruginosa</i> PA14 wild-type control and the <i>phoB</i> mutant under low and high phosphate conditions.	80
Figure (24) Swimming motility of <i>P. aeruginosa</i> PAO1 wild-type, $\Delta pvdD$, $\Delta pchE/F$ and the double knock out $\Delta pvdD/pchE/F$	81
Figure (25) pExoT-gfp expression in the <i>P. aeruginosa</i> PAO1 wild-type, $\Delta pvdD$, $\Delta pchE/F$ and the double knock out $\Delta pvdD/pchE/F$ as measured by FACS analysis.	81
Figure (26) proposed model for PhoB-dependent regulation of the biofilm and virulence phenotype.	85
Figure (27) Phenotypes controlled by the Pho regulon and <i>flgF</i> mutant.....	88
Figure (28) Proposed model for the regulation of biofilm formation and TTSS by the Pho regulon and FlgF.	90

List of Tables

Table (1) List of plasmids and constructs used in this work.....	34
Table (2) List of primers used in this study	34
Table (3) Putative Pho boxes and the distance from the start codon in the promoter region of the genes PA3258, PA2567 and PA1727.....	61
Table (4) Real-time quantitative RT-PCR for transcription of the genes PA3258, PA2567 and PA1727 in the <i>P. aeruginosa</i> strain PA14.....	63
Table (5) List of up-regulated genes in the PA14 <i>flgF</i> mutant, as compared to PA14 wild-type.	75
Table (6) List of down-regulated genes in the PA14 <i>flgF</i> mutant, as compared to PA14 wild-type.....	77

1 INTRODUCTION

1.1 *Pseudomonas aeruginosa* – an opportunistic pathogen

Pseudomonas aeruginosa is a motile Gram-negative rod-shaped bacterium. It is a highly versatile and adaptable organism that is able to grow, survive and persist under a broad range of environmental conditions and can be found in most of the natural environments including terrestrial, marine and freshwater habitats (Green *et al.*, 1974; Goldberg, 2000; Spiers *et al.*, 2000). The ecological achievements of *P. aeruginosa* can be attributed to its large genomic and metabolic potential. With 6.3 million base pairs, encompassing 5570 predicted open reading frames (ORF), the genome of type strain PAO1 contains a high proportion of transcriptional regulators or two-component regulatory systems (~ 10 %) (Stover *et al.*, 2000), which facilitate adaptive physiological responses (Mathee *et al.*, 2008).

Moreover, *P. aeruginosa* causes infections in a wide range of eukaryotic organisms including plants, invertebrates and vertebrates rendering it an important opportunistic pathogen (Vasil, 1986; Rahme *et al.*, 1995; Walker *et al.*, 2004). While it rarely infects healthy individuals, it is the most common Gram-negative bacterium involved in nosocomial infections of immunodeficient patients (Bodey *et al.*, 1983). Infections of *P. aeruginosa* can either be acute or chronic (Furukawa *et al.*, 2006). Acute infections such as ventilator-associated pneumonia and urinary tract infections are characterized by rapid bacterial growth, eventually followed by sepsis, and if untreated, frequently death of the host (Parrillo *et al.*, 1990). In contrast, during chronic infections the bacteria persist within the host for years, e.g. in chronic cystic fibrosis lung infections (Lee *et al.*, 2005; Smith *et al.*, 2006; Jelsbak *et al.*, 2007).

1.2 Chronic *P. aeruginosa* infections

P. aeruginosa has an exclusive role in chronic infection in cystic fibrosis (CF) airways. CF is a well-characterized, severe autosomal recessive disorder in the Caucasian population, with an incidence of 1 in 2500 live births (Collins, 1992; Ratjen and Doring, 2003). The disorder is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Boucher, 2002). *CFTR* regulates the movement of chloride (Cl^-), sodium (Na^+) and water from the basolateral to the apical surface of the epithelial cells (Boucher, 2002). The defect of chloride ions transport leads to disruption of the cell's salt/water balance resulting in increased viscosity of various secretions including the mucus layer within the lung (Boucher, 2002).

P. aeruginosa grows in biofilm-like structure within dehydrated viscous airway mucus, while cycles of infection and inflammation lead to progressive deterioration of lung function, which determines the course and prognosis in most CF patients (Koch and Hoiby, 1993; Ratjen and Doring, 2003). Chronic lung infections represent the leading cause of morbidity and mortality in CF patients despite even intensified antimicrobial therapy (Govan and Deretic, 1996; Tummeler and Kiewitz, 1999; Lyczak *et al.*, 2000; Driscoll *et al.*, 2007).

1.3 Biofilm development

The bacterial biofilm development is widely accepted to take place through a number of steps discovered by microscopic analysis of the biofilm communities over time (Yahr and Parsek, 2006). As demonstrated in Fig (1), these steps include initial attachment. The cells undergo a reversible attachment via the flagellated pole and an irreversible attachment, in which the cells make a more stable interaction with the surface via its long axis (Caiazza and O'Toole, 2004; Toutain *et al.*, 2007). The adhesion organelles such as: flagella, type IV pili and CupA fimbriae have been shown to be essential for initial attachment of the bacteria to the surfaces and are required for the biofilm maturation (O'Toole and Kolter, 1998; Vallet *et al.*, 2001; Klausen *et al.*, 2003a). Subsequently, the attached bacteria aggregate, grow and proliferate to form microcolonies. Within the microcolonies the bacteria lose the flagella and start to produce alginate and the extracellular matrix, which consists of several biopolymers including proteins, polysaccharides, and nucleic acids. This matrix plays a crucial role in the structural development of the biofilms (Ryder *et al.*, 2007). The microcolonies continue to grow and form the mature biofilm, in which mushroom-like colonies separated by water-filled channels are developed, followed by detachment during which bacteria actively leave the biofilm.

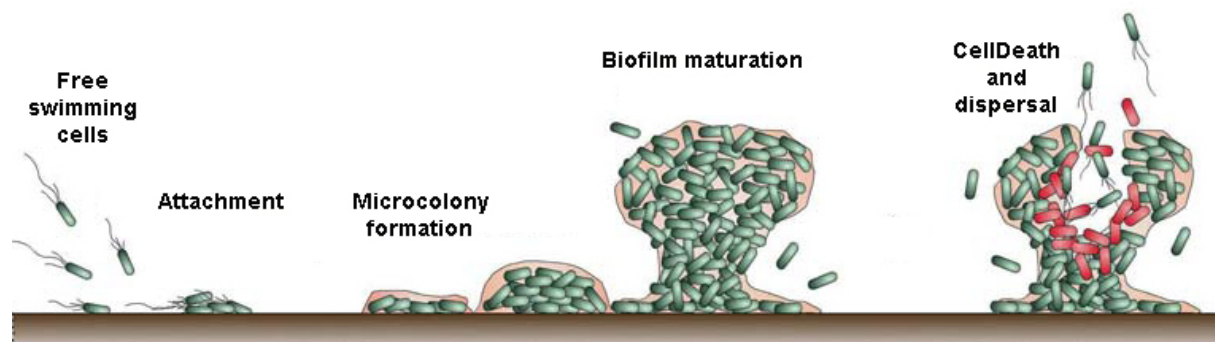


Figure (1) Stages of the biofilm development.

Swimming bacteria attach to an abiotic surface, the attached bacteria aggregate and form microcolonies. Subsequently, they lose the flagella and start to produce the extracellular matrix. In mature biofilms mushroom like structures are separated by water-filled channels. Finally, the biofilm is dispersed by death of subpopulations of cells and detachment of planktonic bacterial cells from the biofilm. Modified from (McDougald *et al.*, 2008).

1.4 Resistance of biofilms

Inside the biofilms, structured communities of microorganisms are enclosed in a self-produced extracellular matrix of polymeric compounds, which is important for the biofilm resistance phenotype (Hall-Stoodley *et al.*, 2004; Ryder *et al.*, 2007). One of the most important aspects of bacterial biofilm infections is their remarkable resistance to antimicrobial agents and the host immune response (Hoiby, 2002; VanDevanter and Van Dalfsen, 2005). *P. aeruginosa*, growing in biofilms, exhibit an increased resistance to antibiotics as compared to their free swimming counterparts (up to 1000 times) (Nickel *et al.*, 1985; Prosser *et al.*, 1987; Costerton *et al.*, 1999; Stewart, 2002). Several protective mechanisms have been proposed to explain this biofilm resistance, including impaired penetration of antibiotics into the biofilm matrix, reduced growth rates of the bacteria within the biofilm, and an induced expression of specific resistance genes (Costerton, 1999; Costerton *et al.*, 1999; Gilbert *et al.*, 2002; Mah *et al.*, 2003). In addition, biofilm bacteria undergo a number of physiological, metabolic and phenotypic changes leading to the emergence of population diversity (Gilbert *et al.*, 2002). The appearance of morphological diversity in the biofilm seems to guarantee the survival of persisting bacteria even under environmental perturbations, which would be able to re-establish a biofilm afterwards (Boles *et al.*, 2004).

1.5 Factors contributing to biofilm formation

1.5.1 Motility and attachment organelles

P. aeruginosa is motile via a single polar flagellum and exhibits three distinct types of motility namely: swimming, swarming and twitching. Swimming motility is mediated by the flagellum while swarming motility requires both flagellum and type IV pili, however twitching motility is type IV pili dependent (Bradley, 1980; Whitchurch *et al.*, 1991; Darzins, 1994; Feldman *et al.*, 1998; Kohler *et al.*, 2000; Rashid and Kornberg, 2000). All types of motility are required for biofilm development.

Flagella

The bacterial flagellum is a long, thin filament that protrudes from the cell body and consists of three main structures: the basal body, the hook and the filament. As shown in Fig. (2), the basal body is embedded in the cytoplasmic membrane and consists of three rings (the cytoplasmic membrane supramembrane (MS) ring, the peptidoglycan (P) ring and the outer membrane lipopolysaccharide (L) ring) surrounding a rod that traverses the periplasm. The hook is a flexible universal joint between the basal body and the filament, and is exposed to the surface. The long, helical shaped filament is composed of up to 20,000 subunits of polymerized flagellin monomers capped by the flagellar cap, FliD, which is a mucin adhesin (Arora *et al.*, 1998; Chevance and Hughes, 2008).

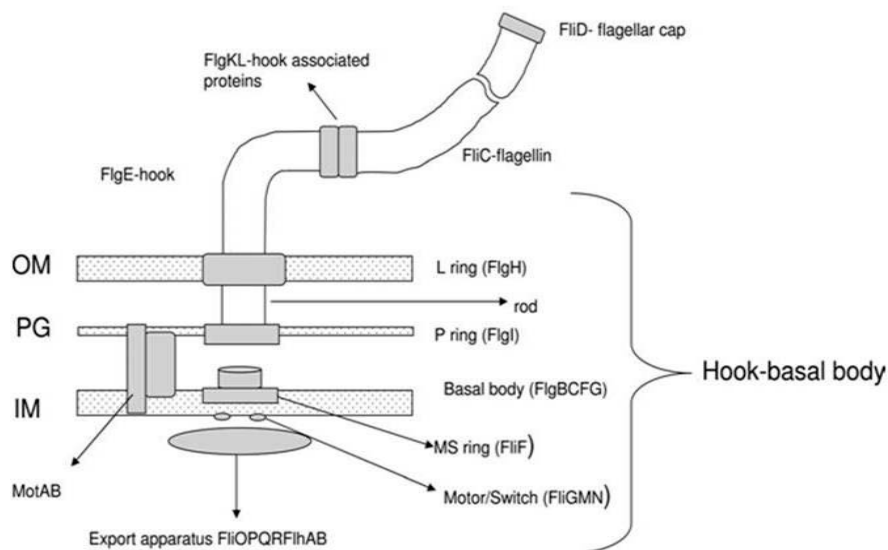


Figure (2) Schematic diagram of the flagellar structure in *P. aeruginosa*.

The diagram shows the different flagellar proteins and their locations (Jyot and Ramphal, 2008).

1. INTRODUCTION

Flagella are required for the initial attachment of the bacteria to surfaces and they are involved in biofilm maturation (O'Toole and Kolter, 1998). The flagellar stators MotAB and MotCD have been shown to be important for early biofilm formation stages in *P. aeruginosa* and to influence reversible attachment. Mutants in these flagellar stators were found to be defective in biofilm formation in both static or flow cells systems although they didn't show an altered swimming motility phenotype (Toutain *et al.*, 2007). Moreover, the cytoplasmic protein SadB has been shown to inversely regulate swarming motility and biofilm formation by altering flagellar reversal rates (Caiazza *et al.*, 2007). It has been suggested that SadB, as well as the flagellar stators, could be part of a regulatory system involved in the inverse control of swarming motility and biofilm formation in response to an unknown signal (Toutain *et al.*, 2005; Toutain *et al.*, 2007). However, another study of biofilm development in *P. aeruginosa* showed that flagella are not involved in attachment and that initial microcolony formation occurs by clonal growth (Klausen *et al.*, 2003a).

Pili and type I fimbriae

The best characterized pili are the type IV pili, which are composed of a helical polymer of the PilA subunit. The type IV pili are localized to the cell poles and mediate most of the adhesive properties of *P. aeruginosa* (Hahn, 1997). Moreover, type IV pili appear to be required for host colonization and biofilm formation. Type IV pili promote cell aggregation and formation of microcolonies (Watson *et al.*, 1996; O'Toole and Kolter, 1998; Vallet *et al.*, 2001). *P. aeruginosa* has also three sets of type I fimbriae, known as CupA, CupB and CupC fimbriae, assembled by the chaperone usher pathway (Vallet *et al.*, 2001). The fimbriae, especially CupA, have been demonstrated to be important for adherence to abiotic surfaces, autoaggregation of small colony variants (SCV) and biofilm formation in *P. aeruginosa* (Vallet *et al.*, 2001; Vallet *et al.*, 2004; Kulasekara *et al.*, 2005; Meissner *et al.*, 2007; Ruer *et al.*, 2007).

1.5.2 Exopolysaccharides

P. aeruginosa produces different types of exopolysaccharides which are important and major components of the biofilm matrix. These exopolysaccharides include Alginate, Psl polysaccharide, and Pel polysaccharide. Alginate is associated with the mucoid strains (Govan and Deretic, 1996) while the Psl and Pel are associated with the non-mucoid strain (Friedman and Kolter, 2004b).

Alginate

P. aeruginosa produces an exopolysaccharide known as alginate. This capsular polysaccharide is overproduced in mucoid *P. aeruginosa* strains (Govan and Deretic, 1996). Alginate is a high molecular weight, acetylated polymer composed of nonrepetitive monomers of β -1,4 linked L-guluronic and D-mannuronic acids. Alginate-producing variants are regularly isolated from chronically infected CF lungs due to mutations in the negative regulator MucA (Martin *et al.*, 1993; Govan and Deretic, 1996; Ramsey and Wozniak, 2005). This polymer acts as an important adherence factor and a physical barrier, which can protect the bacteria from phagocytic clearance, it scavenges oxygen free radicals, and increases resistance of the biofilm towards antimicrobial agents and the host immune defense. Thus, alginate strongly influences the ability of mucoid strains to persist and establish chronic infections in the CF lung (Krieg *et al.*, 1988; Simpson *et al.*, 1989; Pier *et al.*, 1990; Govan and Deretic, 1996). The overproduction of acetylated alginate results in important architectural and morphological changes within the biofilm, which increase the resistance properties of the biofilm (Hentzer *et al.*, 2001; Nivens *et al.*, 2001; Wozniak *et al.*, 2003; Stapper *et al.*, 2004; Ryder *et al.*, 2007). However, it has been demonstrated that in non-mucoid *P. aeruginosa* strains such as PAO1 and PA14, alginate is not the major component of the biofilm matrix (Wozniak *et al.*, 2003).

PEL and PSL polysaccharides

Additional important polysaccharides implicated in biofilm formation, especially in the non-mucoid *P. aeruginosa* strains such as PAO1, ZK2870 and PA14, are the polysaccharides encoded by the *pel* and the *psl* operon. These polysaccharides constitute the major components of the extracellular polysaccharide matrix (Friedman and Kolter, 2004a; Jackson *et al.*, 2004; Matsukawa and Greenberg, 2004; Ma *et al.*, 2006; Ma *et al.*, 2007).

The first gene locus, *pel*, is an operon consisting of 7 genes (PA3058 to PA3064), which encode for Pel polysaccharide biosynthetic proteins. Pel is a glucose-rich matrix polysaccharide polymer with unknown structure. It was found to be involved in pellicle formation and maintenance of biofilms in *P. aeruginosa* PA14 strain (Friedman and Kolter, 2004a, b). Moreover, it was demonstrated that the Pel polysaccharide is important for attachment in a non-piliated *P. aeruginosa* PAK strain (Friedman and Kolter, 2004b; Vasseur *et al.*, 2005). The cytoplasmic protein SadB

1. INTRODUCTION

has been shown to inversely regulate biofilm formation by altering the expression of Pel polysaccharide (Caiazza *et al.*, 2007).

The second polysaccharide synthesis locus, *psl*, is an operon composed of 15 genes (PA2231 to PA2245), encoding the Psl biosynthetic machinery. Psl is composed of a mannose-rich and galactose-rich polysaccharide; however the exact Psl structure has not been clarified. It was demonstrated that Psl exopolysaccharide is an important determinant of different stages of biofilm development in *P. aeruginosa*. During attachment, Psl is anchored on the cell surface, promoting cell-cell interactions and assembly of a matrix, which holds bacteria in the biofilm and on the surface. Furthermore, Psl was shown to be involved in differentiation and maturation of non-mucoid *P. aeruginosa* biofilms as the expression of Psl exopolysaccharide is localized at the center of developing biofilm microcolonies. Psl exopolysaccharide was shown to accumulate on the periphery of 3-D-structured microcolonies, resulting in a Psl matrix-free cavity in the microcolony center. At the dispersion stage, swimming cells appear in this matrix cavity (Overhage *et al.*, 2005; Ma *et al.*, 2006; Ma *et al.*, 2009). Intracellular level of the small signaling molecule bis-(3',5')-cyclic-dimeric-guanosine monophosphate (c-di-GMP), the Wsp chemosensory system, as well as to the GacS/GacA/RsmZ have been demonstrated to control the expression of both *psl* and *pel* operons in *P. aeruginosa* (Hickman *et al.*, 2005; Ventre *et al.*, 2006; Lee *et al.*, 2007).

1.5.3 Rhamnolipids

P. aeruginosa produce biosurfactants such as rhamnolipids, which are synthesized by enzymes of the *rhlABC* operon. Rhamnolipids were shown to be required for biofilm formation as they promote microcolony formation in the initial phase of the biofilm, are involved in maintaining void spaces and channels in mature biofilms and are also implicated in biofilm dispersion (Davey *et al.*, 2003; Boles *et al.*, 2005; Pamp and Tolker-Nielsen, 2007).

1.6 Intercellular signaling (quorum sensing)

Bacteria within biofilms are able to communicate with each other via cell-to-cell communication systems also known as quorum sensing (QS). QS is defined as cell density-dependent signaling and is mediated via self generated extracellular signal molecules (Lazdunski *et al.*, 2004; Williams *et al.*, 2004; Joint *et al.*, 2007; von

Bodman *et al.*, 2008). Quorum sensing allows bacteria to monitor their population density by sensing extracellular concentration of self generated diffusible signal molecules known as autoinducers (Bassler, 2002; Lazdunski *et al.*, 2004). The concentration of the autoinducers increases along with bacterial cell density and once a certain concentration threshold is reached, the autoinducers bind to receptors (regulators), resulting in alteration of the expression of target genes (Keller and Surette, 2006). QS signal molecules control the production of numerous secreted virulence factors, including proteases and secondary metabolites (Pesci *et al.*, 1999; Rumbaugh *et al.*, 2000; Venturi, 2006). Furthermore, QS has been shown to regulate biofilm formation via an influence on the production of polysaccharides, rhamnolipids and extra-cellular DNA (eDNA) in the biofilm matrix (Davies *et al.*, 1998; Allesen-Holm *et al.*, 2006; Nakamura *et al.*, 2008; de Kievit, 2009). Two N-acyl homoserine lactone (AHL)-dependent QS systems – *las* and *rhl* – are present in *P. aeruginosa*, involving the two signaling molecules N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-homoserine lactone (C4-HSL) (Fuqua *et al.*, 1994; Latifi *et al.*, 1995; Latifi *et al.*, 1996). These two Lux-type QS systems are hierarchically organized and directly or indirectly regulate more than 10% of the *P. aeruginosa* genome (Schuster *et al.*, 2003; Smith and Iglewski, 2003; Wagner *et al.*, 2003; Schuster and Greenberg, 2006). While QS regulation itself is dependent on high cell densities, the *las* and *rhl* systems are additionally closely connected to a third 2-alkyl-4-quinolone (AQ)-dependent QS system, involving the *Pseudomonas* quinolone signal (PQS) and its precursor HHQ (2-heptyl-4-quinolone) (Pesci *et al.*, 1999; Dubern and Diggle, 2008). In addition, there are further factors that manipulate the activity of the QS systems, for instance, the two component systems GacA/GacS and PhoB/PhoR as well as the transcriptional regulators PqsR and Vfr (Albus *et al.*, 1997; Reimann *et al.*, 1997; Cao *et al.*, 2001; Jensen *et al.*, 2006).

1.7 Siderophores

Although iron is the most abundant transition metal on earth, bioavailable iron Fe^{2+} levels are low in aerobic environments. In the biological fluids of the hosts, iron is sequestered by iron transport proteins (e.g. transferrin and lactoferrin), which have high affinity for Fe^{3+} , while intracellular iron (mostly present as Fe^{2+}) is either engaged in the prosthetic group of iron proteins or stored in the ferritin core (Ratledge and Dover, 2000; Smith *et al.*, 2004; Bullen *et al.*, 2006). To overcome this iron

limiting growth conditions, *P. aeruginosa* produces iron chelators (siderophores) such as pyoverdine and pyocheline (Poole and McKay, 2003).

Pyoverdine consists of a dihydroxyquinoline fluorescence chromophore to which an acyl side-chain and a peptide chain are attached (Poole and McKay, 2003). Pyoverdine can obtain iron from the host's transferrin and lactoferrin (Xiao and Kisaalita, 1997). Pyoverdine from *P. aeruginosa* is required for virulence in animal models of disease (Meyer *et al.*, 1996; Takase *et al.*, 2000). Moreover, pyoverdine-dependent iron transport is also essential for biofilm development (Visca *et al.*, 2007).

Pyochelin is the second important siderophore produced by *P. aeruginosa*. It is a low molecular weight thiazoline derivative [2(2-*o*-hydroxy-phenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid] (Cox *et al.*, 1981). Despite the low affinity of pyocheline for Fe^{3+} in aqueous media, it is effective at promoting iron uptake in *P. aeruginosa* (Cox, 1980; Crosa and Walsh, 2002). Moreover, pyochelin is capable of chelating other transition metals and is responsible for the uptake of both Co^{2+} and Mo^{4+} into *P. aeruginosa* cells (Visca *et al.*, 1992). Pyocheline plays a minor role in pathogenicity due to its moderately low affinity to Fe^{3+} (Meyer *et al.*, 1996; Takase *et al.*, 2000).

1.8 Two component systems

Microbes that are able to adapt to and survive in diverse environments devote a significant portion of their genome to signal transduction and gene regulation (Ventre, 2007). Sequence analysis of the *P. aeruginosa* PAO1 genome revealed that about 10 % of the open reading frames encode for putative transcriptional regulators or two-component system, including 64 sensors and 72 response regulators (Stover *et al.*, 2000). This amazing number of regulatory proteins enables the bacteria to sense and to orchestrate adaptive response to a wide variety of changing environmental conditions (Rodrigue *et al.*, 2000; Venturi, 2006; Gooderham and Hancock, 2009).

The two-component regulatory systems (TCS) function as signal transducers and regulators to directly translate environmental signals into adaptive regulatory responses. TCSs usually consist of two proteins: a membrane-bound sensor histidine kinase and a cytoplasmic response regulator (Hoch, 2000; Stock *et al.*, 2000; Szurmant *et al.*, 2007). The sensor kinase consists of a signal recognition domain (input domain) attached to an autokinase transmitter domain. The sensor kinase is

able to detect one or more environmental signals via its input domain, and subsequently a conserved histidine residue in its cytoplasmic transmitter domain undergoes autophosphorylation. The transfer of the phosphoryl group to a conserved aspartate residue in the receiver domain of the response regulator protein subsequently modifies the activity of the output domain, which activates the signaling pathway and generates an appropriate cellular response. The output domain of the response regulator is often a helix-turn-helix DNA binding domain, which activates the transcription of target genes. The response regulator output domains can also contain enzymatic functions such as GGDEF and EAL domains, catalyzing the production or degradation, respectively, of the second messenger c-di-GMP (Galperin, 2004; Romling and Amikam, 2006).

1.8.1 Phosphate (PHO) regulon

The phosphate (Pho) regulon comprises the two-component system PhoR / PhoB and is essential for bacterial adaptation to phosphate starvation (Wanner, 1993). PhoR is an integral membrane signaling histidine sensor kinase that senses environmental inorganic phosphate (P_i) levels by interaction with the ABC-type phosphate-specific transport (Pst) system and a protein called PhoU. These proteins are encoded together within the *pstSCAB-phoU* operon (Rao and Torriani, 1990; Lamarche *et al.*, 2008). Under high phosphate conditions the sensor kinase, PhoR, is inhibited from phosphorylation and hence activation of its cognate partner response regulator PhoB. However, under phosphate limiting conditions PhoR is directed to phosphorylate PhoB and thus to promote the interaction of PhoB with Pho boxes, scattered among the chromosome. Phosphorylated PhoB activates the transcription of those genes preceded by Pho boxes. Members of the Pho regulon typically encode for proteins important for phosphate assimilation or metabolism in *E. coli* (Wanner, 1996), but in *P. aeruginosa* it is also important in QS and virulence (Jensen *et al.*, 2006).

There is increasing evidence that there is a link of the Pho regulon and the expression of virulence traits (Aoyama *et al.*, 1991; Ruberg *et al.*, 1999; Slater *et al.*, 2003; Lamarche *et al.*, 2005; von Kruger *et al.*, 2006; Lamarche *et al.*, 2008). The PhoR / PhoB TCS was shown to participate in the regulation of the invasion genes in *Salmonella enteric* serovar Typhimurium (Lucas *et al.*, 2000; Baxter and Jones, 2005). The PhoB or PhoR homologs in other bacteria such as *Agrobacterium*

1. INTRODUCTION

tumefaciens (Mantis and Winans, 1993), *Corynebacterium glutamicum* (Kocan *et al.*, 2006) and *Vibrio cholerae* (von Kruger *et al.*, 2006) were demonstrated to be necessary for virulence. Mutation of the *phoB* or *phoR* gene homologs leads to reduction of the virulence features in these strains.

Furthermore, the Pst system in *E.coli*, has been shown to influence virulence. Mutations in the Pst system resulted in impaired or even avirulent phenotypes in the tested infection models via influence on numerous virulence aspects such as resistance against the bactericidal effect of serum, acidity and cationic antimicrobial peptides (Harel *et al.*, 1992; Ngeleka *et al.*, 1992; Daigle *et al.*, 1995; Batisson *et al.*, 2003; Lamarche *et al.*, 2005; Buckles *et al.*, 2006). Moreover, proteome analysis of *Edwardsiella tarda* showed that the type three secretion system effector proteins were absent in a *pst* mutant (Rao *et al.*, 2004). These studies underlay that the Pho regulon is important not only for phosphate homeostasis but also for modulation of virulence traits.

The Pho regulon has also been demonstrated to influence biofilm formation. Mutations in the Pst system for instance, were shown to inhibit biofilm formation in *Pseudomonas aerofaciens* and *Pseudomonas fluorescens* (Monds *et al.*, 2001). The Pho regulon was implicated to play a role in regulation of biofilm formation in *Pseudomonas fluorescens* Pf0-1 by controlling the production of the adhesion protein LapA through modulation of c-di-GMP levels via RapA, which has a c-di-GMP phosphodiesterase activity (Monds *et al.*, 2007). The PhoR / PhoB TCS was demonstrated to induce the production of biofilms under phosphate-depleted conditions in the plant pathogen *Agrobacterium tumefaciens* (Danhorn *et al.*, 2004).

In *E. coli* PhoB activity has been demonstrated to be induced in a phosphate independent manner in the absence of the sensor kinase PhoR. The sensor protein CreC, implicated in the regulation of genes involved in carbon catabolism was shown to induce PhoB activity in the absence of PhoR via cross-regulation (Wanner and Wilmes-Riesenbergs, 1992). Acetyl phosphate can also cause activation of PhoB in *E.coli* (Kim *et al.*, 1996). Furthermore, mutations in the Pst system lead to constitutive expression of the Pho regulon independent on phosphate availability (Wanner, 1996).

1.9 C-di-GMP

Environmental signals can be transmitted into appropriate bacterial responses by membrane-bound receptors feeding into cytoplasmic second-messenger cascades. Second-messenger systems are capable of integrating many sensory inputs and offer flexibility of recognition and response. It was recognized that the second messenger bis-(3',5')-cyclic-dimeric-guanosine monophosphate (c-di-GMP) is ubiquitous in gram-negative bacteria (D'Argenio and Miller, 2004; Jenal, 2004; Romling *et al.*, 2005; Romling and Amikam, 2006).

Originally, c-di-GMP was discovered and identified by Benziman and co-workers as an allosteric activator of the membrane bound cellulose synthase in *Gluconacetobacter xylinum* (Ross *et al.*, 1987; Weinhouse *et al.*, 1997; Tal *et al.*, 1998). Now, c-di-GMP is known as a bacterial second-messenger molecule that is universally involved in the molecular decision between planktonic motile and sedentary biofilm-associated bacterial lifestyles (Simm *et al.*, 2004; Romling and Amikam, 2006; Hengge, 2009). A rise in c-di-GMP levels commonly favors the sessile mode of life resulting in an increased expression of various factors required for the establishment and maintenance of biofilm communities such as adhesive matrix components, exopolysaccharides and fimbriae. On the other hand a decreased production of c-di-GMP or its degradation usually favors the planktonic lifestyle and leads to an enhanced expression of virulence and motility factors. This correlation has been demonstrated for *Pseudomonas aeruginosa* and many other bacteria (Simm *et al.*, 2004; Gjermansen *et al.*, 2005; Jenal and Malone, 2006; Thormann *et al.*, 2006; Rahman *et al.*, 2007; Verstraeten *et al.*, 2008; Hengge, 2009; Ueda and Wood, 2009).

The cellular levels of c-di-GMP are modulated via several proteins, which contain either GGDEF or EAL, or hybrid GGDEF/EAL domains. Proteins containing a GG(D/E)EF domain exhibit diguanylate cyclase (DGC) activity thus synthesize c-di-GMP by catalyzing the condensation of two molecules of GTP (Simm *et al.*, 2004; Ryjenkov *et al.*, 2005; Jenal and Malone, 2006). In contrast, proteins containing an EAL domain possess c-di-GMP-specific phosphodiesterase (PDE) activity and hydrolyze c-di-GMP into linear 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG). Moreover, proteins containing a HD-GYP domain are able to degrade c-di-GMP to GMP (Christen *et al.*, 2005; Schmidt *et al.*, 2005; Tamayo *et al.*, 2005; Ryan *et al.*,

1. INTRODUCTION

2006). Synthesis and degradation of c-diGMP by DGC and PDE are shown in Fig. (3). In *P. aeruginosa* several GGDEF and / or EAL domain containing proteins (38 in PAO1 and 37 in PA14) have been identified (Kulasakara *et al.*, 2006). This large number of proteins in a single bacterial species indicates flexibility, specificity and complexity of c-di-GMP signaling (Hengge, 2009).

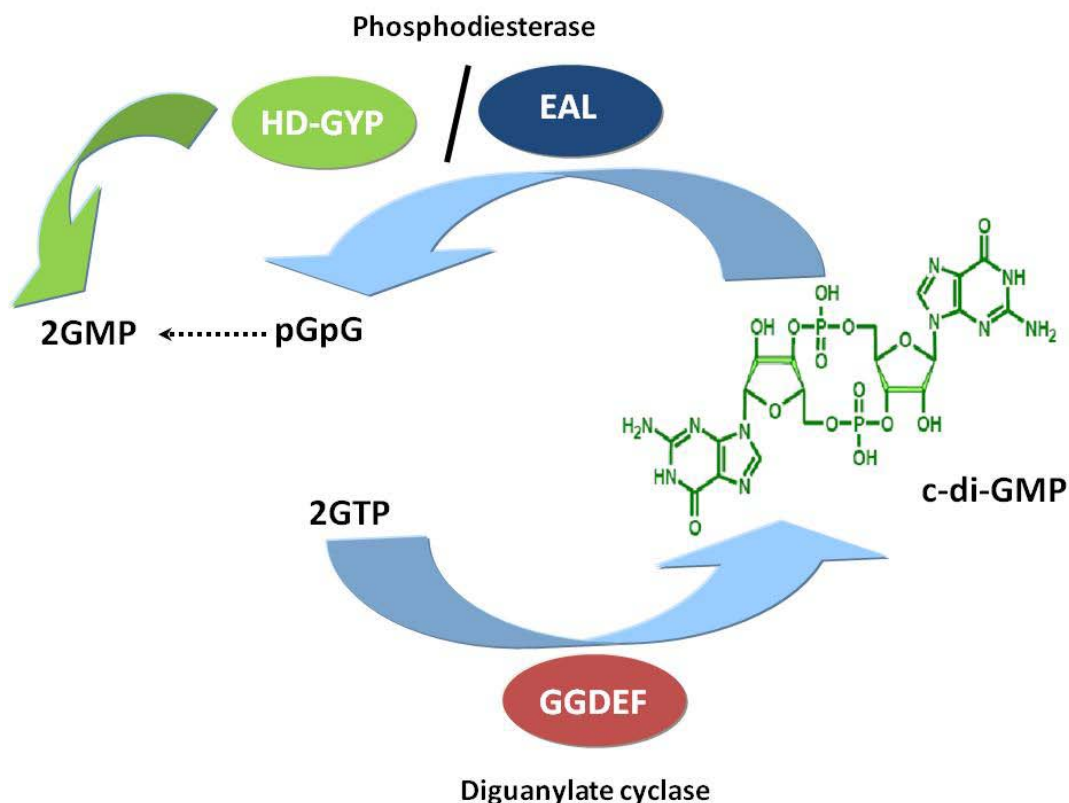


Figure (3) Synthesis and degradation of c-di-GMP.

Cellular levels of c-di-GMP are controlled by (GGDEF, EAL and HD-GYP) domain containing proteins harboring DGC or PDE activity. GGDEF diguanylate cyclases synthesize c-di-GMP from two GTP molecules, whereas c-di-GMP is degraded into the linear form pGpG by EAL phosphodiesterases, which is further degraded to two GMP molecules. HD-GYP phosphodiesterases hydrolyze c-di-GMP to pGpG and are able to further degrade pGpG into GMP. Modified from (Romling *et al.*, 2005).

1.9.1 Downstream targets of c-di-GMP

In *P. aeruginosa*, several proteins were found to be downstream targets of c-di-GMP. It was shown that c-di-GMP binds to PilZ domain effector proteins such as PilZ and Alg44 proteins (Alm *et al.*, 1996; Merighi *et al.*, 2007). C-di-GMP can bind to the flagellar regulator, FleQ, (Hickman and Harwood, 2008). PelD has also been verified to be a downstream target of c-di-GMP providing another link between c-di-GMP signaling and exopolysaccharide regulation (Lee *et al.*, 2007). Recently it has been

found that some RNA binding proteins (regulators) modulate the global cellular c-di-GMP levels by controlling the expression of several GGDEF and EAL proteins, by binding to and changing their mRNA levels (Sudarsan *et al.*, 2008). For example, in *E. coli*, the carbon storage regulator (CsrA) controls the expression of GGDEF and EAL proteins, especially the GGDEF proteins YcdT and YdeH by binding to their mRNA (Jonas *et al.*, 2008).

1.9.2 Impact of c-di-GMP on the virulence phenotype

C-di-GMP was shown to be involved in regulation of several cellular components such as flagella, fimbriae, type three secretion system and exopolysaccharides which have been shown to play a dual role in biofilm formation and virulence of *P. aeruginosa*. The GGDEF and EAL domain containing protein MorA has been shown to be involved in regulating biofilm formation in *P. putida* and *P. aeruginosa* (Choy *et al.*, 2004). In *P. aeruginosa*, FleQ, the flagella expression regulator and Pel exopolysaccharide repressor was shown to be inhibited by binding to c-di-GMP resulting in a reduction of flagella expression and an increase of the exopolysaccharides production, hence promoting biofilm formation (Hickman and Harwood, 2008). Moreover, the expression of the *pel* and *psl* gene loci was shown to be linked to the intracellular level of the small signaling molecule cyclic-di-GMP (Lee *et al.*, 2007). The intracellular c-di-GMP levels were shown to influence CupA fimbriae expression in the autoaggregative small colony variants (SCV) of a clinical *P. aeruginosa* strain. Expression of these CupA fimbriae was dependent on functional PA1120 and *morA* genes both encoding proteins harboring a GGDEF domain (Meissner *et al.*, 2007). Expression of type IV fimbriae has been demonstrated to be linked to FimX, the PDE containing protein (Huang *et al.*, 2003; Kazmierczak *et al.*, 2006). Alginate biosynthesis was found to be enhanced by c-di-GMP in mucoid *P. aeruginosa*. The protein Alg44 is required for alginate production and has a PilZ domain, which binds c-di-GMP. Furthermore, *mucR* (PA1727) encodes for a c-di-GMP-synthesizing enzyme and was demonstrated to be involved in regulation of alginate production (Merighi *et al.*, 2007; Hay *et al.*, 2009). It was exposed that certain virulence-associated traits such as biofilm formation and type three secretion system mediated cytotoxicity are controlled by multiple DGCs and PDEs through alterations in c-di-GMP levels in *P. aeruginosa* (Kulasakara *et al.*, 2006).

1.10 Type three secretion system of *P. aeruginosa*

The type three secretion system (TTSS) is a major virulence and cytotoxicity determinant of *P. aeruginosa* (Nicas and Iglewski, 1984; Sawa *et al.*, 1999; Holder *et al.*, 2001). The TTSS mediates translocation of toxins from the bacteria directly into the eukaryotic host cells. The TTSS delivers four toxins (effectors), ExoS, ExoT, ExoY, and ExoU (Frank, 1997). In addition, PcrV, PopB and PopD are secreted proteins by the TTSS. They are important for the translocation of toxins to host cells (Vallis *et al.*, 1999). TTSS toxins are thought to be translocated into host cells through a channel formed by the PopB, and PopD oligomer. The third translocation protein PcrV is required for the membrane insertion of PopD (Goure *et al.*, 2004). The structure of the TTSS injectisome is demonstrated in Fig. (4). TTSS toxins work together to disrupt the host immune response, to inhibit phagocytosis, to promote tissue destruction and to impair wound healing (Finck-Barbancon *et al.*, 1997; Geiser *et al.*, 2001). The cytotoxins ExoS and ExoT inhibit phagocytosis by disrupting actin cytoskeletal rearrangement, focal adhesins and signal transduction cascades important for phagocytic function (Barbieri and Sun, 2004; Kipnis *et al.*, 2006). The effectors ExoY and the ExoU have adenylate cyclase and phospholipase activities, respectively (Kulich *et al.*, 1994; Yahr *et al.*, 1996; Finck-Barbancon *et al.*, 1997; Yahr *et al.*, 1998; Sato and Frank, 2004).

The TTSS of *P. aeruginosa* is controlled by a complex regulatory network of specific regulators (the positive transcriptional activator ExsA, which is the master regulator of the type III secretion system (Frank, 1997), the anti-activator ExsD, the anti- anti-activator ExsC, and the secreted anti-anti-anti-activator ExsE) (McCaw *et al.*, 2002; Dasgupta *et al.*, 2004; Rietsch *et al.*, 2005; Urbanowski *et al.*, 2005). Moreover, the TTSS responds to global regulators for instance: Vfr, quorum sensing (Wolfgang *et al.*, 2003; Hogardt *et al.*, 2004; Bleves *et al.*, 2005) and two-component systems (RetS, LadS, RocAR, and CopRS) (Goodman *et al.*, 2004; Ha *et al.*, 2004; Laskowski *et al.*, 2004; Kuchma *et al.*, 2005; Ventre *et al.*, 2006; Yahr and Wolfgang, 2006).

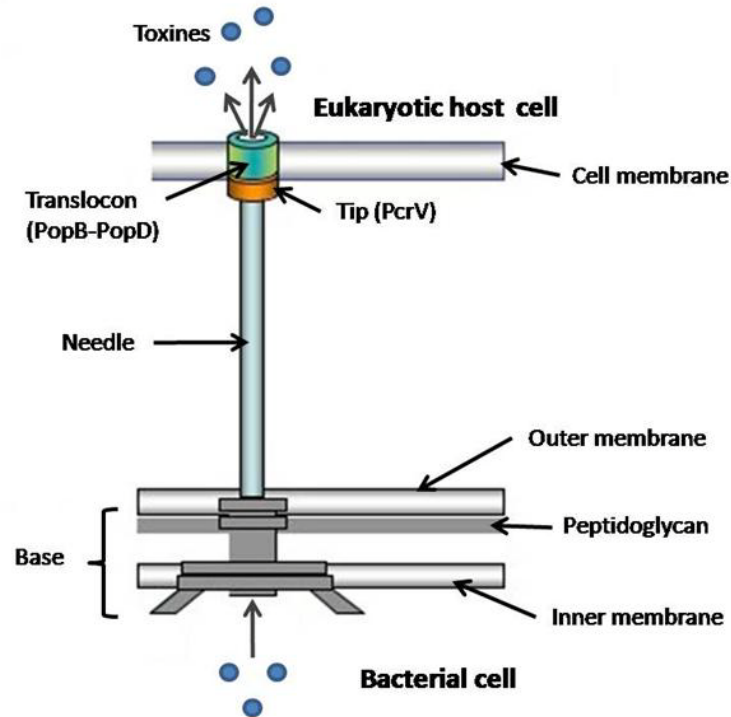


Figure (4) Schematic structure of the type three secretion injectisome in *P. aeruginosa*.

TTSS injectisome is composed of a basal body, which is usually topped by a needle-like structure that protrudes from the bacterial surface. The needle is thought to function as the conduit for protein translocation. *P. aeruginosa* delivers 4 toxins known as ExoS, ExoT, ExoY and ExoU along the TTSS injectisome directly into the host cell. Delivery of these toxins also requires a set of type three secreted proteins called translocators (PopB and PopD). These proteins assemble into a translocation pore in the target-cell membrane. PcrV at the needle tip is required for the membrane insertion of PopD. Modified from (Mota, 2006).

1.11 Aim of the work

Morbidity and mortality of chronic *P. aeruginosa* infections, especially in CF lung patients, is associated with biofilm formation. Within the biofilm, bacteria are resistant to most of the therapeutic agents and the host immune response leading to persistence of the infection. Therefore, it is important to investigate the mechanisms that regulate biofilm formation to develop new therapeutic strategies for the treatment of chronic *P. aeruginosa* infections. The switch between the motile planktonic and sedentary biofilm-associated bacterial lifestyle was found to be highly regulated by environmental signals (Jackson *et al.*, 2002; Stanley and Lazazzera, 2004; Banin *et al.*, 2005). However, the knowledge about the mechanisms by which these environmental signals are sensed and translated into biofilm formation is limited (Monds *et al.*, 2007). It has been shown that phosphate is an important environmental signal molecule and that the Pho regulon inhibits biofilm formation in *P. aureofaciens* and *P. fluorescens*. Interestingly, the inhibition of biofilm formation was mediated by PhoB-dependent activation of a downstream phosphodiesterase, which decreases the intracellular c-di-GMP levels in *P. fluorescens* (Monds *et al.*, 2001; Monds *et al.*, 2007). It has been demonstrated that the Pho regulon influences virulence of *P. aeruginosa* via regulation of QS (Jensen *et al.*, 2006). In this study we investigated a link between the Pho regulon and intracellular signaling via c-di-GMP in *P. aeruginosa*, and we proved that the Pho regulon influences the virulence phenotype (represented by biofilm and TTSS) at least partially via c-di-GMP.

2 MATERIALS AND METHODS

2.1 Bacterial strains

2.1.1 *Pseudomonas aeruginosa*

PA14 strain

P. aeruginosa PA14 wild-type and isogenic transposon mutants were obtained from Harvard Medical School, USA.

(<http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi>).

PAO1 strain

P. aeruginosa PAO1 wild-type and isogenic transposon mutants were obtained from the University of Washington, Genome center, USA.

(<http://www.gs.washington.edu/labs/manoil/libraryindex.htm>).

The pyoverdine and pyochelin knock out mutants of *P. aeruginosa* PAO1; $\Delta pvdD$, $\Delta pchE/F$ and $\Delta pvdD/pchE/F$ were kindly provided by Cornelis P., Brussel, Belgium.

2.1.2 *Escherichia coli*

E. coli DH5 α

This strain was used as an intermediate recipient for pUCP20 constructs designed in this study. The plasmid containing strain was grown in presence of 100 μ g/ml ampicillin.

E. coli BL21(DE3)

This strain was the final recipient of the pET21a(+) expression vector (Novagen) and used for protein expression. This strain was grown in presence of 100 μ g/ml ampicillin.

2.2 Culture media

2.2.1 Luria Bertani (LB) medium (Sambrook *et al.*, 1989)

LB medium was used for routine cultivation of all bacterial strains. When required, bacteria were streaked onto LB plates with 1.5% agar.

LB medium (1 L)

Bacto-trypton	10 g
Yeast extract	5 g
NaCl	5 g
Distilled H ₂ O up to	1000 ml

2.2.2 Columbia blood agar plates (Becton Dickinson)

Columbia blood agar plates were used to cultivate the bacterial strains as an alternative to LB agar plates. Especially, these plates were used to detect morphotypic variants of *P. aeruginosa* strains.

2.2.3 DeMoss minimal medium (Frank and Demoss, 1959)

Minimal medium was used to grow *P. aeruginosa* strains for biofilm quantification, TTSS expression and transcriptome profiling. The medium contained either (0.8 mM) or (4.0 mM) di-potassium hydrogen phosphate, which represented the low or high phosphate conditions respectively.

DeMoss Minimal medium (1 L)

D-alanine	10 g
Glycerol	20 ml
K ₂ HPO ₄	variable
MgCl ₂ .6H ₂ O	4.06 g
Distilled H ₂ O up to	1000 ml
Final pH was adjusted to 7.4 with 1 N NaOH	

Directly before inoculation, a solution of ferric citrate was added, to obtain a 50 µM final concentration of ferric citrate.

2.2.4 Tryptone soymeal broth (TSB)

This medium was used for growing *P. aeruginosa* under conditions inducing TTSS expression (Hornef *et al.*, 2000). The medium contained either (0.8 mM) or (4.0 mM) di-potassium hydrogen phosphate, which represented the low or high phosphate conditions respectively.

TSB medium (1 L)

Peptone from casin	17.0 g
Peptone from soyameal	3.0 g
D(+)-Glucose	2.5 g
NaCl	5.0 g
K ₂ HPO ₄	variable
Glycerol	1 %
Nitrilloacetic acid	0.01 M

2. MATERIALS AND METHODS

Glutamic acid	0.1 M
Distilled water Up to	1000 ml

The pH was adjusted to 7.3 with 1 N NaOH

2.3 Buffers and solutions

2.3.1 1x PBS (Phosphate-buffered saline)

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄ ·2H ₂ O	1.77 g
KH ₂ PO ₄	0.24 g
H ₂ O up to	1000 ml

The pH was adjusted to 7.4

2.3.2 Agarose Electrophoresis buffers

TAE buffer

Tris	40 mM
EDTA	2.5 mM
Acetic acid	20 mM

The pH was adjusted to 8.0

DNA loading buffer

Xylencyanol	0.25 g
Bromophenol blue	0.25 g
Ficoll 400 (Sigma)	25.00 g
EDTA (Roth)	1.46 g
H ₂ O up to	100 ml

The solution was filtered and sterilized by autoclaving.

2.3.3 SDS-PAGE buffers

4x Protein Loading Buffer

H ₂ O	9.9 ml
0.5 M Tris Base (pH6.8)	1.2 ml
Glycerin	4.0 ml
10% (w/v) SDS solution	8.0 ml
2-β-Mercaptoethanol	0.8 ml
5% (w/v) Bromphenolblue	0.1 ml

2. MATERIALS AND METHODS

1x SDS Running Buffer

Tris Base	25 mM
Glycine	192 mM
SDS	0.1 % (w/v)

The pH was not adjusted

Colloidal Coomassie blue staining solutions

Staining solution A

Ammoniumsulfat	10 % (w/v)
(85%) o-Phosphoric acid	2 % (w/v)

Staining solution B

5% (w/v) Coomassie brilliant blue G-250

Distaining solution

Ethanol	30 % (v/v)
Acetic acid	10 % (v/v)

2.3.4 Western blot buffers

Blot buffer

Tris-Base	25 mM
Glycine	190 mM
SDS	0.1 % (w/v)
Methanol	20 % (v/v)

The pH was adjusted to 8.5

10x TBS (Tris-buffered saline)

NaCl	80 g
Tris-base	30 g
KCl	2 g
H ₂ O up to	1000 ml

The pH was adjusted to 8.0

1x TBS-T

10x TBS	100.0 ml
Tween20	1 g
H ₂ O up to	1000 ml

2.4 Plasmids and constructs

All plasmids, vectors and constructs used in this study are listed in Table 1.

Table (1) List of plasmids and constructs used in this work

Plasmid	Character	Reference
pUCP20	A shuttle vector for cloning in <i>E. coli</i> and <i>P. aeruginosa</i> ; carb ^R	(West <i>et al.</i> , 1994)
pUCP20: <i>phoBR</i>	A construct for expression of both genes <i>phoB</i> and <i>phoR</i> ; carb ^R	This study
pUCP20: <i>rapA</i>	A construct for expression of the gene <i>rapA</i> ; carb ^R	This study
pUCP20: <i>flgF</i>	A construct for expression of the gene <i>flgF</i> ; carb ^R	This study
pExoT:GFP	A construct harbor the promoter and the N terminal sequences of <i>exoT</i> preceeding <i>gfp</i> gene ; cm ^R	(Hornef <i>et al.</i> , 2000)
pET21a (+)	A vector for expression of proteins in <i>E. coli</i> ; amp ^R	Novagen
pET21a(+) <i>rapA</i>	A construct for expression of the protein RapA in <i>E.coli</i> ; amp ^R	This study

2.5 Primers

The primers used in this work are listed in Table 2 and were designed according to the *Pseudomonas* Genome Database v2 (www.pseudomonas.com) using Vector NTI software (Invitrogen). These oligonucleotides were synthesized by the company MWG Biotech (Germany).

Table (2) List of primers used in this study

Primer	Sequence	Purpose
PA3258_EcoRI_F1	GCAT <u>GAA</u> TCGAGCTGATGAAGGAGAAGAC	Cloning
PA3258_HindIII_R1	GCATA <u>AAG</u> CTTAAGCTCTGAATGGATCTGGCG	Cloning

2. MATERIALS AND METHODS

<i>PhoBR</i> _EcoRI_F1	GATC <u>GAATTCT</u> ACGCCGTTGTGTCACATAC	Cloning
<i>PhoBR</i> _HindIII_R1	GATC <u>AAGCTT</u> AAAAGCGCAAAGAGGATCAG	Cloning
<i>flgF</i> _SalI_F	CTAGG <u>TCTGACT</u> GTTTTTCGAATTCTGGCAC	Cloning
<i>flgF</i> _HindIII_R	CTAGA <u>AAGCTT</u> ATCATCTTCACGTGCAGCTCG	Cloning
PA3258_F	GACCTGCACTGCCTGTTCCA	RT-PCR
PA3258_R	GTTCCAGTTCGCTCAGCCGT	RT-PCR
PA2567_F	CCTCGCCCTGCTGAATACCA	RT-PCR
PA2567_R	TCTTCCTGTTGCTGCCGTGG	RT-PCR
PA1727_F	GCGGTGCTGGTCATCGTGAT	RT-PCR
PA1727_R	TGGGCAGCTTGGTCAGGTTG	RT-PCR
PA1580_F	TCCAAGGGCGAGCCGATGATGTA	RT-PCR
PA1580_R	AGGCGATGCAGGCGAACGGATTG	RT-PCR
<i>rapA</i> _HisTag_F	CCTG <u>CATATGG</u> TGAAATACGGGGCCGGTTGC	Expression
<i>rapA</i> _HisTag_R	CATG <u>AAGCTT</u> GGCGGCCTCGATCAGGTGCA	Expression

2.6 Preparation of permanent cultures

Bacteria were inoculated in 3 mL LB medium in presence of the appropriate antibiotics when required. The culture was incubated overnight at 37°C with shaking. Vials containing a 1:1 mixture of bacterial culture and 80% glycerol (650 µl each) were frozen in liquid nitrogen, and subsequently stored at -70°C.

2.7 Static quantitative biofilm assay

Biofilm assays were performed in polyvinylchloride (PVC) 96 well plates (microtest III flexible assay plate, Becton Dickinson Labware, USA) as previously described by O'Toole and Kolter, 1998 with some modifications. Briefly, mid-log phase LB bacterial

2. MATERIALS AND METHODS

cultures (6-8 hours) were washed twice with DeMoss medium and adjusted to an OD₆₀₀ of 0.1 in DeMoss minimal medium under low or high phosphate conditions. 100 µl per well were incubated in the 96-well plates at 37 °C for 24 hours. Afterwards the wells were extensively washed with distilled water to remove unbound bacterial cells. The plate was air dried and the attached bacterial biofilm was stained with 150 µl crystal violet (0.1 % in water) for 20 minutes. The plate was washed several times with 200 µl distilled water to remove the excess of crystal violet that was not bound to the bacterial biofilm. The plate was dried and the crystal violet that stained the surface associated biofilm was dissolved in 200 µl of de-staining solution (95 % Ethanol). 150 µl from each well were transferred to a fresh flat bottom micro-titer plate and the absorption was read at 550 nm by a SunriseTM plate reader (Tecan Trading AG, Switzerland). Readings from no less than six wells for each strain or condition were used to calculate the average biofilm mass.

2.8 Biofilm assay and 3D-imaging by confocal scanning laser microscope

To assess biofilm formation ability of *P. aeruginosa*, we scanned the biofilm formed at the bottom of a 96 well plate by means of confocal laser scanning microscope. Precultures were grown in tubes overnight at 37 °C on a shaking unit. On the next day precultures were diluted to an OD of 0.02 in LB and 100 µl were transferred to a well of the sterile 96-well µClear[®] microplate (Greiner Bio-One). The plate was covered with an air permeable BREATHsealcover foil (Greiner Bio-One) and incubated at 37 °C in an incubator with a humid atmosphere. After 24 h the bacteria were stained with the LIVE/DEAD[®] BacLight Bacterial Viability Kit (Molecular Probes/Invitrogen). 50 µl of a diluted staining solution was added to each well resulting in a final concentration of 1.4 µM Syto9 and of 8.3 µM propidium iodide (PI). Microscopy was performed after 72 h using an Olympus Fluoview 1000 system equipped with a 20x air objective.

2.9 Congo red binding assay

Congo red is a dye able to bind to neutral polysaccharides or polysaccharides that contain either -1,3- or -1,4- glucopyranosyl units. The more polysaccharides are produced by a given strain, the less the residual Congo red will be present in the culture supernatant. The assay was done according to Lee *et al.* (Lee *et al.*, 2007) with some modifications. Briefly, a fresh colony from the strain to be tested was

2. MATERIALS AND METHODS

inoculated in 3 ml LB and incubated for 6-8 h at 37 °C and 180 rpm. These bacterial precultures were centrifugated and the pellet was washed twice with 3 ml DeMoss minimal medium before inoculating the main culture (5 ml fresh DeMoss medium under low or high phosphate conditions). The bacteria were incubated for 24 h at 37 °C and 180 rpm. The OD₆₀₀ was standardized to 2 and the bacterial content of each culture was collected from 2 ml by centrifugation. The bacterial pellets were resuspended in 1 ml of 40 mg/ml Congo red in 1% tryptone and incubated for 2 h at 37 °C and 250 rpm. The bacteria and bound Congo red were collected by centrifugation and the amount of Congo red remaining in the supernatant was determined by measuring the absorbance of the supernatant at 490 nm.

2.10 Swimming motility assay

Two µl from a log phase culture were stab inoculated into the middle of a soft LB agar plates (0.3 % agar). Then, the plates were incubated overnight at 37 °C and the swimming zone was measured in cm.

2.11 Flow cytometric analysis of ExoT expression

For the determination of ExoT expression, the plasmid pExoT-GFP, which harbors the promoter and the N-terminal sequence of the effector protein ExoT in front of the reporter gene *gfp* (kindly provided by M. Hogardt, LMU Munic) was introduced in the respective *P. aeruginosa* strains by electroporation. A single colony from a fresh plate of pExoT-GFP transformed bacteria was inoculated in 3 ml LB medium supplemented with 750 µg/ml chloramphenicol and incubated overnight at 37 °C with shaking. The cells were collected by centrifugation and washed twice with DeMoss medium. The bacteria were inoculated in 3 ml DeMoss minimal medium under high or low phosphate conditions with an initial OD₆₀₀ of 0.05. The cultures were incubated at 37 °C for 22 h with shaking. The bacteria were adjusted to an OD₆₀₀ of 0.5 in phosphate-buffered saline (PBS), then diluted 1:100 in the same buffer and subjected to fluorescence-activated cell sorter (FACS) cytometry analysis to determine the average intensity of fluorescence of GFP-producing bacteria. The fluorescence data were collected for 50,000 cells using a FACS calibur cytometer (BD Biosciences). Summit software (Dako Colorado) was used for data analysis.

2.12 Pyoverdine and pyochelin measurement

A single colony from a fresh plate of the *P. aeruginosa* strain to be tested was inoculated in 3 ml LB medium and incubated overnight at 37 °C with shaking. The bacterial cells were collected by centrifugation and washed twice with DeMoss medium. The main cultures were prepared by inoculation of the washed bacteria in 3 ml DeMoss medium under low or high phosphate conditions with an initial OD₆₀₀ of 0.05 and incubated for 22 h at 37 °C with shaking. Pyoverdine and pyochelin were measured in the culture supernatant with the microtitre plate fluorometer (MFX Microtiter[®] Plate Fluorometer, DYNEX Technologies, USA). The fluorescence was determined by exciting the culture supernatant at 400 nm for pyoverdine and 355 nm for pyochelin, the emission was measured at 460 nm for pyoverdine and at 440 nm for pyochelin (Xiao and Kisaalita, 1998; Kim *et al.*, 2003).

2.13 Nucleic acids techniques

2.13.1 Isolation of genomic DNA

Genomic DNA was isolated from bacteria using DNA Tissue Kit (Qiagen) according to the manufacturer's protocol. Briefly, from 5 ml overnight culture, 1 ml was centrifuged for 5 minutes at 5000 rpm. The pellet was washed 3 times each with 1 ml distilled H₂O and centrifuged for 1 min at 13000 rpm. These washing steps were performed to remove the exopolysaccharides matrix, which may interfere with the DNA isolation by blocking the silica gel membrane of the columns. The bacteria were resuspended in the lysis buffer and applied to the column. The DNA binds to the silica gel membrane of the column. During washing steps of the column, the DNA purity increased and finally the pure DNA was eluted from the column by H₂O. The quality of the DNA was tested in 1 % agarose gel and the quantity of the DNA was determined by UV-absorption using the NanoDrop ND-1000 spectrophotometer (Peqlab).

2.13.2 Isolation of plasmid DNA

Plasmid DNA was isolated from 3 ml overnight bacterial cultures using QIAprep. Spin Miniprep Kit (Qiagen, Germany) according to the manufacturer's instructions. The integrity of the plasmids and constructs were proven by restriction enzymes digestion and agarose gel electrophoresis, and sequencing.

2. MATERIALS AND METHODS

2.13.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed according to the standard method of Sambrook *et al.* (Sambrook *et al.*, 1989). Agarose-gels were prepared at concentrations of 1-2 % agarose in electrophoresis buffer (1x TAE). The mixture was boiled in a microwave oven to dissolve the agarose. After cooling down of the agarose solution to 60 °C, GelStar was added (4 µl/100 ml) and the solution was poured into the gel tray. GelStar is an intercalating dye that has fluorescent emissions under UV light (Nucleic Acid Gel Stain, 10,000x DMSO, BMA, Rockland, USA). The agarose gel solution solidified at room temperature. For electrophoresis a horizontal electrophoresis apparatus (BioRad) was used. Before loading on the gel, samples were mixed with DNA loading buffer. Electrophoresis was run at a voltage of 80 V, supplied by Consort E815 (LTF) power supply. To estimate the size of nucleic acids, SmartLadder (Eurogentec) or GeneRuler 50 Bp marker (Fermentas) were run parallel to the samples in the same gel. A multiimageTM light cabinet (Biozyme) was used to photograph the gel under UV-light.

2.13.4 Cloning of the PCR products

PCR amplification of gene sequences

In order to amplify the genes of interest, the corresponding cloning primers mentioned in Table (2) were used in the PCR with PA14 wild-type genomic DNA as template.

The PCR reaction conditions were set up as indicated below:

PCR mixture	
<i>Pfu</i> 10X Rxn buffer (Promega)	5 µl
dNTPs	4 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
Template DNA (10 ng)	1 µl
<i>Pfu</i> polymerase (Promega)	0.5 µl

2. MATERIALS AND METHODS

Sterile water	34.5 μ l
Total reaction volume	50 μ l

PCR Reaction		
Initial denaturation	95 °C	2 min
Denaturation	95 °C	1 min
Annealing	Variable	20 sec
Extension	72 °C	Variable
Final extension	72 °C	5 min
Cooling and storage	4 °C	∞

Purification of PCR products

Amplified PCR products were purified using Qiagen PCR purification kit (Qiagen) according to the manufacturer's protocol.

Cloning vectors

PCR products were cloned into either the shuttle vector pUCP20 (West *et al.*, 1994) to complement corresponding *P. aeruginosa* mutants, or into the vector pET21a(+) (Novagen) to express the protein of interest in *E. coli*. followed by protein purification.

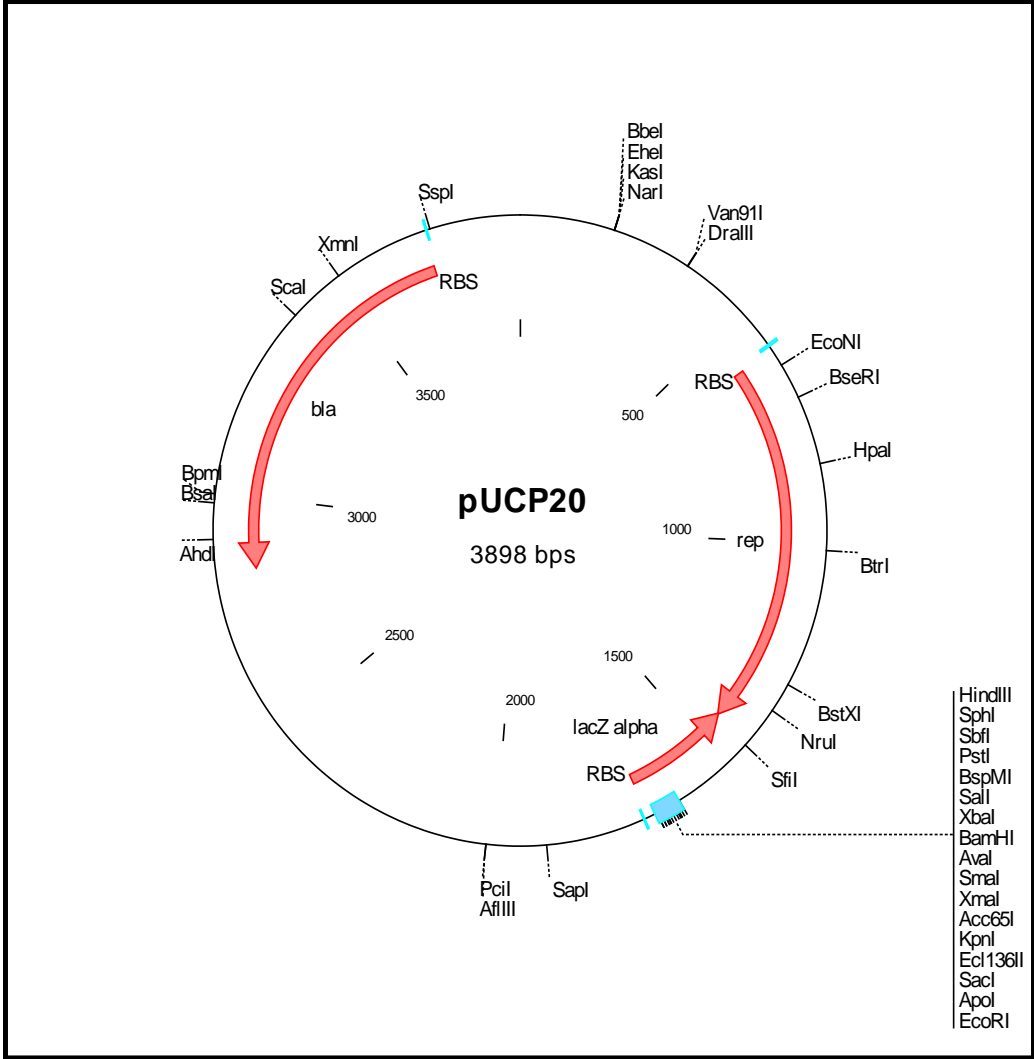


Figure (5) Restriction map of the shuttle vector pUCP20.
The map shows the multiple cloning site within the *LacZ* gene.

2. MATERIALS AND METHODS

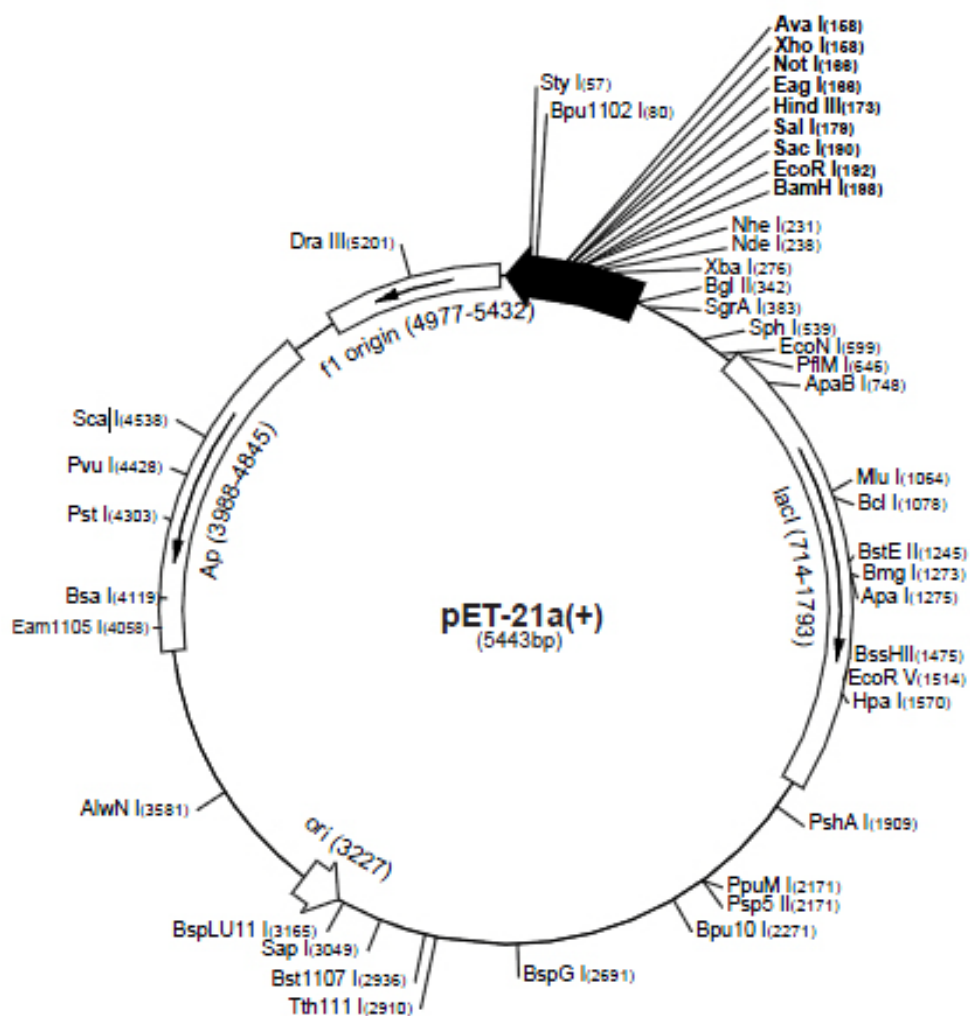


Figure (6) Restriction map of the expression vector pET21a(+) (Novagen).

The map shows the multiple cloning site, the origin of replication (ori) and the ampicillin resistant gene.

2. MATERIALS AND METHODS

Restriction digestion of PCR products and plasmids

Purified PCR products and vectors were cut with 10 units of suitable restriction enzymes (Fermentas) at 37 °C for 2 hours. Then, enzymes were heat-inactivated by heating the samples for 15 minutes at 65 °C.

Purification of restriction products

Restricted PCR fragments and restricted vectors were run on a 1 % agarose gel. Subsequently, DNA fragments of interest were excised from the gel and purified using the DNA and Gel Band Purification Kit (Amersham, Biosciences, UK) according to the manufacturer's instructions.

Ligation of vector and insert

The restricted purified PCR fragment was subsequently ligated into the restricted purified pUCP20 vector. A ratio of 1 : 3 linearized vector : digested PCR product were incubated overnight at 16 °C in presence of 1 unit of T4 Ligase (Roche, Basel, Switzerland) and 1X T4 Ligase buffer (Roche). The ligation product was transformed into *E. coli* DH5α by electroporation.

Preparation of electrocompetent *E. coli*

A single colony of *E. coli* DH5α from a fresh LB agar plate was inoculated into 10 ml LB medium and incubated overnight at 37 °C with shaking. The main culture was prepared by inoculation of 2X 250 ml LB medium with 2.5 ml from the preculture. The bacteria were incubated at 37 °C and 180 rpm until the OD₆₀₀ 0.6 - 0.9 (log phase growth) was reached. Then, cultures were removed from the incubator and placed on ice for 15 min. Cells were harvested by centrifugation in 500 ml centrifuge tubes at 4 °C and 4500 rpm for 15 min. The bacterial pellet was gently resuspended in 200 ml H₂O. The centrifugation was repeated and the cell pellet was softly resuspended in 50 ml H₂O. After centrifugation, the bacterial pellet was gently resuspended in 100 ml 15 % glycerol solution. Centrifugation and the glycerol solution washing step were repeated, followed by resuspension of the bacterial pellet in 6 ml glycerol solution and distribution into 150 µl aliquots. Finally, aliquots were frozen in liquid nitrogen and stored in -70 °C.

Electroporation of *E. coli*

Two microliters of ice incubated ligation products were carefully mixed with 48 µl of freshly thawed electrocompetent *E. coli* and were loaded into a chilled 2 mm gap

2. MATERIALS AND METHODS

width electroporation cuvette (Bio-Rad). In another cuvette, 50 µl of electrocompetent cells were placed to serve as a negative transformation control. In a third cuvette, 2 µl of the vector and 48 µl of electro-competent cells were mixed as described above and used as positive transformation control. Electroporation was carried out using GenePulserXcellk Electroporation System (Bio-Rad) at 25 µFD, 400Ω, and 2.5 kV (time constant = 6-8 msec). Immediately after electroporation, 1 ml of a pre-warmed LB medium was added to the cuvettes. The mixture of each cuvette was transferred into 1.5 ml Eppendorf tube and incubated for 1 h at 37 °C. After incubation, 100 µl of the mixtures were plated separately on LB agar plates containing 100 µg/ml of ampicillin to select for the *E.coli* cells transformed with the vector (positive control) or with the constructs. The plates were incubated overnight at 37 °C. The constructs were purified from the selected colonies using a QIAGEN Plasmid Mini Kit, and submitted to sequencing (GATC Biotech, AG, Germany). Subsequently, the pUCP20 constructs were transformed by electroporation to different *P. aeruginosa* mutants.

Preparation of electrocompetent *P. aeruginosa*

In order to prepare electrocompetent *P. aeruginosa*, two LB agar plates were inoculated from a fresh colony of *P. aeruginosa* and incubated overnight at 37 °C. The bacteria were collected in a 1.5 ml Eppendorf tube containing 1ml MilliQ water and were mixed by vortexing. The bacteria were centrifuged at 13000 rpm for 1 min. The supernatant was removed and the bacterial pellet was resuspended in 1ml MilliQ water. The centrifugation, removal of supernatant and resuspension of the pellet in 1 ml H₂O were repeated several times to washout the extracellular polysaccharides matrix from the bacteria. Finally, the bacterial pellet was resuspended in 100 µl H₂O.

Electroporation of *P. aeruginosa*

Freshly prepared electrocompetent *P. aeruginosa* were electroporated using the same procedure as mentioned previously for *E. coli* except for the pulse, which was applied at 25 µFD, 200Ω, and 2.45 kV (time constant = 4-6 msec). Transformed *P. aeruginosa* strains were then plated on LB agar plates containing 500 µg/ml carbenicillin. Single colonies from complemented *P. aeruginosa* strains were selected and permanent cultures were prepared as mentioned before.

2.13.5 RNA isolation

RNA isolation was performed using the RNeasy Protect Bacteria reagent, RNeasy Kit and Qias shredder columns (Qiagen). Samples of DeMoss cultures containing 10^9 bacteria were mixed with the same volume of RNeasy Protect Bacteria reagent, and centrifuged at 8000 rpm for 5 min (Top bench centrifuge). The supernatant was discarded and the samples stored at $-70\text{ }^{\circ}\text{C}$. Bacterial pellets were thawed, centrifuged at 8000 rpm for 5 min and the supernatant left was removed. Pellets were then resuspended with 200 μl of TE solution pH 8.0 containing 800 $\mu\text{g/ml}$ Lysozyme and vortexed for 30 s. Samples were subsequently incubated for 10 min at room temperature and vortexed 2 to 3 times during the incubation time. Seven hundred microliters of an RTL buffer solution containing β -mercaptoethanol (10:1 RTL buffer: β -mercaptoethanol) were added to the samples, which were then stored at $-70\text{ }^{\circ}\text{C}$. Thawed samples were loaded onto a Qias shredder column and centrifuged for 2 min at 14000 rpm. Flow through of each sample was then transferred to a gDNA Eliminator spin column (Qiagen) and RNA purification was performed according to the manufacturer's instructions. The concentration of the RNA samples obtained was measured with a NanoDrop ND-1000 spectrophotometer (PecLab). The quantified RNA was either stored for a maximum of 3 days at $-70\text{ }^{\circ}\text{C}$, or directly used for synthesis of cDNA.

2.13.6 cDNA synthesis

The synthesis of cDNA was performed as follows:

Total RNA volume	20 μl
Random hexamer primers (300 ng/ μl , Invitrogen)	4 μl
Thermocycler temperature paused at $70\text{ }^{\circ}\text{C}$	

Annealing step:

$70\text{ }^{\circ}\text{C}$	10 min
$25\text{ }^{\circ}\text{C}$	10 min

The tubes were removed from the thermocycler and were placed immediately on ice

Thermocycler temperature paused at $25\text{ }^{\circ}\text{C}$

2. MATERIALS AND METHODS

Master Mix	1 rxn
5X 1st Strand Buffer (invitrogen)	10 µl
100 mM DTT (Invitrogen)	5 µl
10 mM dNTP (each)	3 µl
RNAse free H ₂ O	3 µl
SuperScript II (Invitrogen)	5 µl
pre-mix total vol. (without RNA template)	26 µl

Carefully added to each sample (Final reaction volume: 50 µl)

Reverse transcription step:

25 °C 10 min

37 °C 60 min

42 °C 60 min

70 °C 15 min

Ten microliters of sterile water were added to each PCR reaction tube, followed by a 20 µl addition of 1N NaOH. Tubes were then incubated for 30min at 65 °C in a thermocycler to denature the RNA. cDNA neutralization was performed by adding 20 µL of a 1N HCl solution in each reaction tube.

Purification of cDNA was then carried out using a QIAquick PCR Purification Kit (QiaGen), and according to the manufacturer's instructions. The concentration of the purified cDNA was measured with a NanoDrop ND-1000 spectrophotometer (Pepqlab).

2.13.7 Semi-quantitative RT-PCR

Total RNA was isolated from *P. aeruginosa* PA14 which was grown for 22 h in DeMoss minimal medium at low or high phosphate conditions. Approximately 10⁹ bacteria from each tested strain were suspended in equal volume of RNAProtect (Qiagen). The RNA was isolated using RNeasy columns (Qiagen) according the

2. MATERIALS AND METHODS

instructions of the manufacturer. The nucleic acids were treated with DNase I (Roche) and the yield of total cellular RNA was determined by UV-absorption using NanoDrop ND-1000 spectrophotometer (Peglab). In order to quantify mRNA levels of selected genes, cDNA was synthesized from the total RNA using Superscript II kit (Invitrogen) and semi-quantitative PCR was performed using 10 ng cDNA templates for 30 cycles of amplification. RT-PCR primers listed in table (2) were used for amplification of the genes: *rapA*, PA2567, PA1727 and the housekeeping gene *gltA* (PA1580) (coding for citrate synthase). The PCR conditions were:

PCR mixture	
10X colourless buffer (Promega)	5 µl
MgCl ₂	3 µl
DNTPs	4 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
Template DNA (10 ng)	1 µl
<i>GoTaq</i> polymerase (Promega)	0.25 µl
Sterile water	31.75 µl
Total volume	50 µl

PCR Reaction		
Initial denaturation	95 °C	2 min
Denaturation	95 °C	1 min
Annealing	60 °C	20 sec
Extension	72 °C	30 sec
		30 cycles

2. MATERIALS AND METHODS

Final extension	72 °C	5 min
Cooling and storage	4 °C	∞

The PCR products of the genes (*rapA*, PA2527, PA1727 and *gltA*) were loaded onto a 2 % agarose gel, submitted to electrophoresis and compared for the expression level of these genes in the different mutants.

2.13.8 Quantitative real time RT-PCR

The real-time PCR is a method that allows the analysis of the transcriptional regulation of selected genes. Quantitative real time RT-PCR was done with the Applied Biosystem 7500 Real Time PCR system (Applied Biosystems) using QuantiFast™ SYBR® Green RT-PCR Kit (Qiagen) and specific primers optimized to amplify about 200 bp fragments from the genes under investigation (Table 2). SYBR Green is a dye that binds the minor groove of double stranded DNA resulting in increased intensity of the fluorescent emissions, which correlate with the amount of double stranded amplicons. Thus, this method facilitates a quantification of PCR products.

To compare quantitatively the expression levels of the genes of interest, the total RNA samples were isolated from *P. aeruginosa* PA14 wild-type, which were grown for 16, 24 and 40 h in DeMoss minimal medium under high or low phosphate conditions. QuantiFast™ SYBR® Green RT-PCR Kit (Qiagen) was used to amplify the RNA directly in the RT-PCR without cDNA synthesis according the instructions of the manufacturer.

A threshold was set in the linear part of the amplification curve, and the number of cycles needed to reach it was calculated for every gene. Transcription folds of RNA in low phosphate relative to the high phosphate conditions were determined for each time point by using included standard curves for each individual gene and further normalization to the housekeeping gene PA1580. Melting curves established the purity of the amplified band.

After optimization of the primers and the template, the reaction mixture was set up as the following:

2. MATERIALS AND METHODS

Component	96-well plate	Final concentration
2x Quantifast SYBR Green RT-PCR Master Mix	12.5 μ l	1x
Primer A	Variable	1 μ M
Primer B	Variable	1 μ M
QuantiFast RT Mix	0.25 μ l	
Template RNA	Variable	\leq 100 ng/reaction
RNase-free water	variable	
Total reaction volume	25 μ l	

The real time PCR conditions were set as follows:

Step	Time	Temperature	Ramp rate
Reverse transcription	10 min	50 °C	
PCR initial activation step	5 min	95 °C	Maximal
Two step cycling			
Denaturation	10 s	95 °C	Maximal
Combined annealing/extension	30 s	56.2 °C	Maximal
Number of cycles	40		

2.13.9 Transcriptome analysis

The total RNA was isolated using the RNeasy Protect Bacteria reagent, RNeasy Kit and Qiashredder columns from Qiagen as shown previously. The RNA was used for the cDNA synthesis using Superscript II kit (Invitrogen) as demonstrated before. The total

2. MATERIALS AND METHODS

amount of cDNA to be used for an Affymetrix DNA GeneChip should be between 4 and 7 µg.

cDNA fragmentation and terminal labeling

Fragmentation of cDNA was carried out using DNaseI (0,35 U/µg cDNA). The size of the cDNA fragments obtained should be between 50 and 200 base pairs. The fragmented cDNA was then labelled at the 3'-end with biotin-ddUTP (Roche Diagnostics, Indianapolis, IN) using Terminal deoxynucleotidyl transferase (Roche).

The reaction was incubated for 1h at 37 °C using the following reaction mixture:

Reaction mix:	
5x Reaction Buffer (Roche)	12 µl
10x CoCl ₂ (Roche)	6 µl
Biotin-ddUTP (Roche)	1 µl
Fragmented cDNA	39 µl
H ₂ O	1 µl
Terminal Deoxynucleotide Transferase (Roche)	1 µl

The reaction was stopped by adding 2 µl of a 0.5 M EDTA pH 8.0 solution.

The labeling step was then checked by performing a Neutravidin shift test. Five microliters of the labeled cDNA fragments were mixed with 5 µl Neutravidin solution (400 µg Neutravidin/100 µL 50 mM Tris pH 7.0) and incubated for 5 min at room temperature. Samples were then loaded onto a 2 % agarose gel and submitted to electrophoresis for 50 min at 80 V.

Microarray data analysis

For each strain to be tested two *P. aeruginosa* DNA Affymetrix GeneChips were hybridized with 4 – 5 µg of labeled cDNA fragments. The GeneChips were hybridized for 16 hours at 50 °C. After hybridization, the GeneChips were washed, stained with

2. MATERIALS AND METHODS

SA-PE and read using an Affymetrix GeneChip fluidic station and scanner according to Affymetrix standard protocols (Affymetrix, Santa Clara, CA). Normalization of data was performed using the MAS 5.0 Software from Affymetrix (Dotsch *et al.*, 2009).

2.14 Protein techniques

2.14.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts were denatured by heating at 95 °C for 5 min in the presence of the anionic detergent SDS and a reducing agent (β -mercaptoethanol). In this step the proteins were bound to the SDS and became negatively charged, thereby disrupting their subunits. during electrophoresis the proteins were separated according to their molecular weight while migrating towards the anode (Laemmli, 1970).

Protein samples to be analyzed were mixed with protein loading buffer and denatured at 95 °C for 5 min, followed by loading the samples into the wells of the collecting gel. To estimate the molecular weight of the separated proteins, PageRuler™ Prestained Protein Ladder (Fermentus) was loaded in parallel with the samples. Electrophoresis was carried out for approximately 45 min on a Mini Protein II Electrophoresis System (BioRad) and was run in 1x SDS electrode buffer at 75 mA and supplied by Power supply (Biometra).

Separating gel (10 %)	Collecting gel (6 %)	
H ₂ O	2.45 ml	1.15 ml
1.5 M Tris pH 8.8	1.5 ml	-
0.5 M Tris pH 6.8	-	0.5 ml
30% Acrylamide/Bis-acrylamide	1.85 ml	0.34 ml
10% (w/v) SDS solution	0.06 ml	0.020 ml
25% (w/v) APS solution	0.008 ml	0.006 ml
TEMED	0.008 ml	0.004 ml

Acrylamide/Bis-acrylamide:

Rotiphorese Gel 30 (Roth) (30 % (w/v) Acrylamide and 0.8 % (w/v) Bisacrylamide with ratio 37.5:1 respectively).

2.14.2 Colloidal Coomassie blue staining

Protein staining with colloidal Coomassie was performed according to Neuhoff *et al.*, 1988. The colloidal Coomassie Brilliant Blue G-250 was used for staining of SDS-PAGE gels. To carry out the staining, the staining solutions A and B were mixed in a ratio of 98: 2 and then the mixture was shaken vigorously for at least 4 hours in a Schott bottle to form the colloids. Finally, methanol was added to a final concentration of 20 % (v/v). The gel was incubated overnight in the colloidal Coomassie Blue staining solution, followed by de-staining the gel in distilled H₂O until the background was clear (Neuhoff *et al.*, 1988).

The SDS-PAGE gel was documented and photographed with visible light in a Multiimage Light Cabinet (Biozym) and was stored in sealed plastic bags at 4 °C.

2.14.3 Western blot

In order to make further analysis of proteins, which have been previously separated in a polyacrylamide gel, these proteins can be transferred on to a polyvinylidene fluoride (PVDF) membrane without changing the separation pattern using Western blot. We used Western blot analysis to examine the expression of specific proteins in *P. aeruginosa*. For detection of the protein of interest, we used a polyclonal antibody against this protein. The western blot was performed in the following steps:

Semi dry electro blot

For western blot analysis, the proteins that have been separated via SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane (Roth) with the help of the Trans-Blot SD transfer system (Biorad). The membrane was cut to the same gel size then; it was moistened with methanol and equilibrated afterwards together with 6 Whatman paper 3 mm paper of the same size in blot buffer. The gel was also incubated in blot buffer. The SDS-PAGE gel, PVDF-membrane and the Whatman paper were assembled carefully on the cathode plate of the Trans-Blot SD transfer system in the same order as shown in Fig. (7). The transfer of the protein from the gel to the PVDF-membrane (towards the anode) was achieved by applying 120 mA for one gel for 90 minutes.

2. MATERIALS AND METHODS

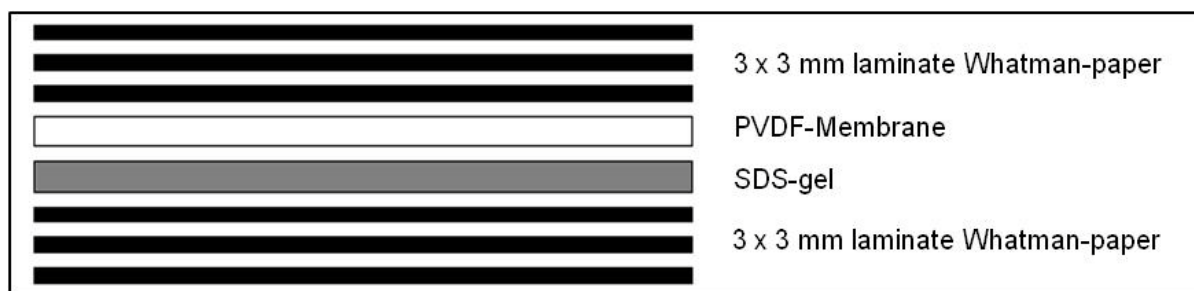


Figure (7) Assembly of the western blot

The SDS-PAGE gel and the PVDF-membrane were carefully put together in between 2 layers of 3 Whatman-paper. The gel was directed towards the cathode plate of the Trans-Blot SD transfer system while the PVDF-membrane was directed towards the anode.

Immunodetection of blotted proteins

The membrane was incubated for 1 h in TBS-T + 10 % skimmed milk powder with shaking to prevent the non specific binding of antibodies to the membrane. Subsequently, the membrane was incubated with the primary antibody (specific to the protein of interest) for 1 h with shaking. The primary antibody was diluted 1:1000 with TBS-T + 10 % skimmed milk powder. The excess of the primary antibody was removed by washing the membrane for 10 min in 15 ml TBS-T, TBS-T + 0.1 M NaCl and TBS-T + 0.5 % (v/v) Triton X-100, respectively. After 1 h incubation with the peroxidase conjugated anti-rabbit IgG antibody (1:2000 diluted in TBS-T + 10 % skimmed milk), the membrane was washed again as mentioned before with an additional washing step in TBS. All steps were performed at room temperature. The analysis was conducted with the help of Lumi-Light Western Blotting Substrate (Roche, Mannheim, Germany) which contains the substrate luminol. The conjugated horseradish peroxidase catalyzes the oxidation of luminol. Immediately following the oxidation, the luminal is in an excited state (intermediate reaction product), which decay to the ground state by emitting light. The emitted light can be documented with a digital camera. The Lumi-Light Western Blotting Substrate was mixed according to manufacturer's instructions, and the washed membrane was incubated for about 1 minute in the mixed solution. The emitted light was detected by the video camera system CDDCamera LAS-100 and Intelligent Dark Box was used (Fujifilm). The recording was done with the software Image Reader 2.5 (Fuji Photo Film Co. Ltd.).

2.14.4 Overexpression of protein in *E.coli*

Time series protein overexpression

The preculture was prepared by inoculation of a single colony from a fresh plate of transformed *E. coli* BL21 (DE3) into 10 ml LB medium containing 100 µg/ml ampicillin and incubated overnight at 37 °C with shaking. The main culture was prepared by inoculation of 2.5 ml from the preculture into 100 ml LB medium supplied with 100 µg/ml ampicillin and incubated overnight at 37 °C with shaking until the OD₆₀₀ reached 0.6 – 0.9. The culture was divided equally into two flasks each contained 50 ml. One of the two cultures was induced with 1 mM Isopropyl-β-D-thiogalactopyranosid (IPTG) while the second culture left without induction. Both cultures were further incubated at 37 °C with shaking. Every 2 h, 1 ml sample was taken out from each culture and the OD₆₀₀ was measured. Cells from each sample were collected by centrifugation and the pellet was resuspended in 50 µl SDS-sample buffer and stored in -20 until use. Samples were thawed and boiled at 95 °C for 15 min before being analyzed. Expression of the protein of interest was assessed by analysis of total cell protein (TCP) on a SDS-PAGE followed by Coomassie blue staining.

Localization analysis of the overexpressed protein

The overexpressed protein was further analyzed for its localization. The preculture was prepared as mentioned above. The main culture was prepared by inoculation of 2.5 ml from the preculture into 50 ml LB medium supplied with 100 µg/ml ampicillin and incubated at (20 °C, 30 °C or 37 °C) until the OD₆₀₀ was 0.6 – 0.9. After induction of the protein expression with 1 mM IPTG, 2x 1 ml sample were taken out at time intervals. These samples named as sample A and sample B were treated as the following:

Sample A was centrifuged for 1 min at 13000 rpm and the pellet was resuspended in 100 µl 1x PBS then mixed with 100 µl 4x SDS-sample buffer. This sample contained the total cellular protein (TCP), it was frozen at -20 °C until use.

Sample B was centrifuged for 1 min at 13000 rpm. The supernatant was transferred to a fresh Eppendorf tube. The proteins in this fraction (Medium fraction) represent the extracellular protein fraction, which was precipitated by 100 µl Trichloroacetic acid (TCA). Subsequently, the pellet was washed twice with 100 µl acetone to remove

2. MATERIALS AND METHODS

the excess TCA. After evaporation of the acetone, the pellet was resuspended in 100 μ l 1x PBS and mixed with 100 μ l 4x SDS-sample buffer then, stored at -20 °C until use.

The pellet of sample B was resuspended in 200 μ l BugBuster-Mix (10x BugBuster reagent, 1:10 diluted with 1x PBS). To degrade the nucleic acids (DNA and RNA), 0.5 μ l Benzonase was added to the mixture. Finally the mixture was incubated with 10 μ l lysozyme (20 mg/ml) at room temperature for 15 min. After centrifugation for 20 min at 13000 rpm at 4 °C, the supernatant was transferred to a new Eppendorf tube and mixed with 100 μ l 4x SDS-sample buffer and stored at -20 °C until use. This fraction represented the soluble protein fraction.

The cell pellets, which represented the insoluble protein fraction, were purified from the inclusion bodies by incubation of the resuspended pellet in 200 μ l BugBuster-Mix and 10 μ l lysozyme (20 mg/ml) for 5 min at room temperature. Subsequently, 1.2 ml diluted BugBuster-Mix (1x BugBuster 1:10 diluted with H₂O) was added to the sample. After centrifugation, the supernatant was removed and the pellet was resuspended in 600 μ l of 1:10 BugBuster. This washing step was repeated 3 times and finally, the pellet was resuspended in 100 μ l 1x PBS and 100 μ l 4x SDS-sample buffer. The different samples were analyzed in a SDS-PAGE.

3 RESULTS

3. RESULTS

Recently, it has been shown that the PHO regulon influences biofilm formation in the two *Pseudomonads*, *P. aureofaciens* and *P. fluorescens*, in response to phosphate limiting conditions (Monds *et al.*, 2001; Monds *et al.*, 2007). It was demonstrated that PhoB activates a downstream protein, RapA, which has an EAL domain and exhibits c-di-GMP phosphodiesterase activity. Low cellular levels of c-di-GMP in turn inhibit the secretion of LapA, a large adhesion protein required for biofilm formation by *P. fluorescens* (Monds *et al.*, 2007). In this thesis we wanted to address the question of whether the Pho regulon also impacts on multicellular behavior in *P. aeruginosa* and if so, whether this is mediated by modulating c-di-GMP signaling.

3.1 The Pho regulon negatively influences biofilm formation in *P. aeruginosa*

We determined the influence of the Pho regulon on biofilm formation in the *P. aeruginosa* strains PA14 and PAO1. We observed an enhanced biofilm formation in a PA14 transposon mutant harboring an insertion in the *phoB* gene. This enhanced biofilm formation was obvious under high and low phosphate medium conditions. The enhanced biofilm formation could be abolished by complementing the *phoB* mutant with the *phoBR* genes *in trans*. Since the disruption of the *pstSCAB-phoU* operon resulted in constitutive activation of the Pho regulon (Wanner, 1993), we tested a PA14 *pstS* transposon mutant for biofilm formation. As expected, the *pstS* mutant exhibited significantly less biofilm under both low and high phosphate conditions as determined by crystal violet staining of attached biofilms in PVC 96 well plate (O'Toole and Kolter, 1998), (Fig. 8 and Fig 9 A).

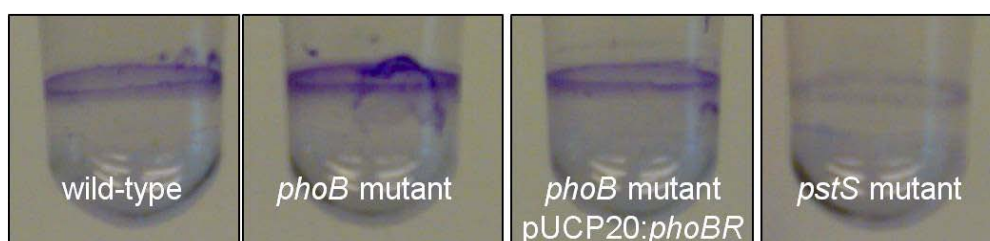


Figure (8) Biofilm formation in PA14 wild-type, *phoB* mutant, *phoB* mutant complemented with pUCP20:*phoBR* and *pstS* mutant.

Bacteria were grown under low P_i DeMoss minimal medium conditions for 24 h at 37 °C. Biofilms on the walls of the PVC 96 well plates were stained with 0.1 % crystal violet (w/v).

3. RESULTS

In the PAO1 strain, we observed an enhanced biofilm formation in the *phoB* mutant. This enhanced biofilm formation was noticed only under low phosphate medium conditions, which was an expected result, because under high phosphate PhoB should be inactive. Complementation of the PAO1 *phoB* mutant with the *phoBR* genes *in trans* could abolish this enhanced biofilm formation under low phosphate medium conditions. Whereas the *pstS* mutant was expected to exhibit less biofilm formation as compared to the *phoB* mutant because of the constitutive expression of the Pho regulon, the *pstS* mutant exhibited an enhanced biofilm formation (Fig. 9B).

Furthermore, overall biofilm formation in PA14 was not significantly influenced by phosphate availability (Fig. 9 A), whereas, in the PAO1 strain high phosphate significantly enhanced biofilm formation (Fig. 9 B). These results suggest that in PA14 PhoB is sufficient to suppress biofilm formation even under high phosphate conditions. Under these conditions PhoB might even be activated via environmental cues other than low phosphate, as it has been described before in *Escherichia coli* (Wanner and Wilmes-Riesenberg, 1992; Kim *et al.*, 1996; Wanner, 1996). In PA14 the *pstS* mutant produced less biofilm than the *phoB* mutant or the wild-type control (Fig. 9 A), again indicating that an active Pho regulon represses biofilm formation. However, in the PAO1 strain the *pstS* mutant produced even more biomass than the *phoB* mutant under low phosphate medium conditions (Fig. 9 B). This result underscores the finding that in PAO1 the availability of phosphate enhances biofilm formation independent of the inhibiting activity of the Pho regulon.

3. RESULTS

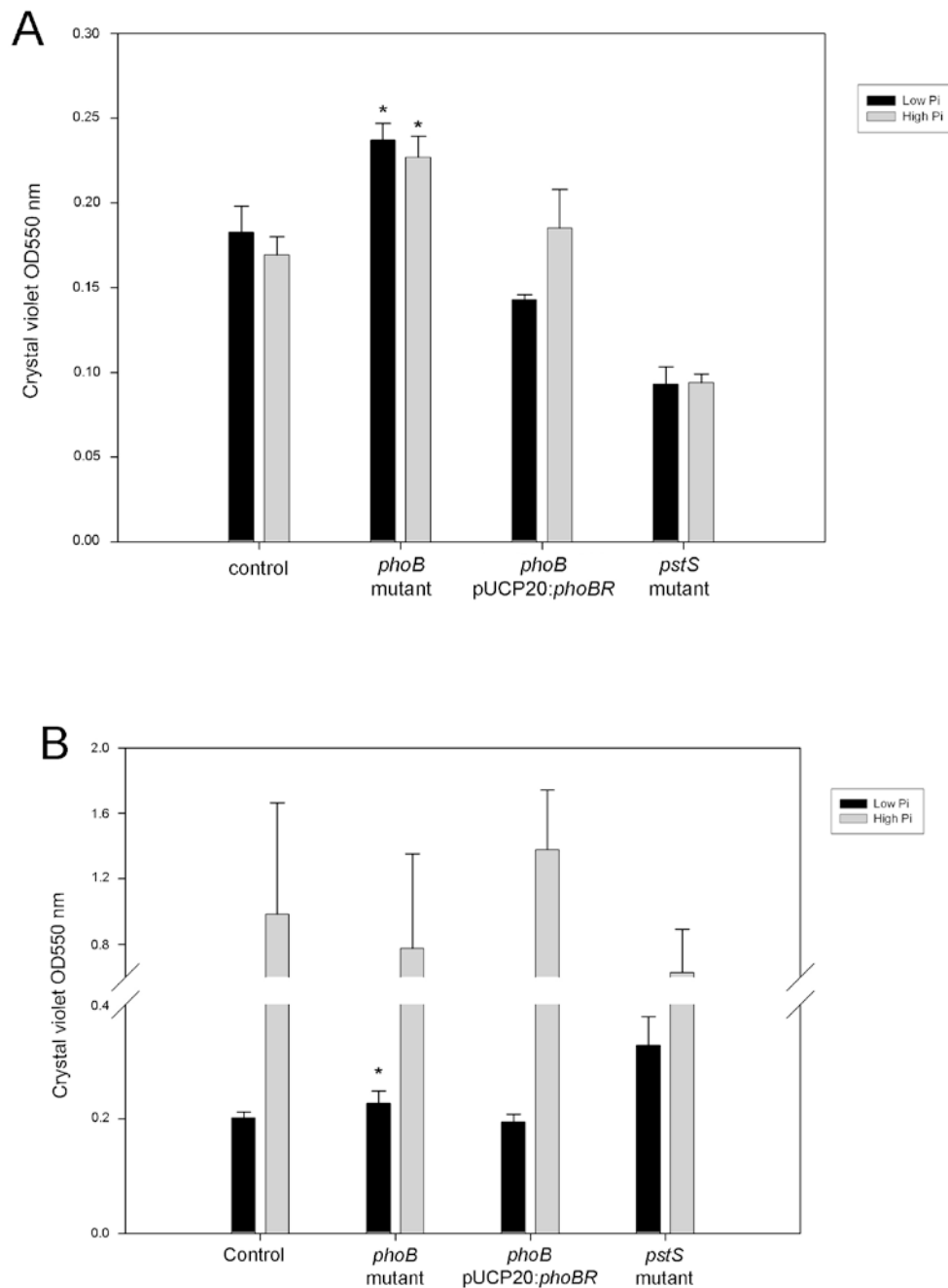


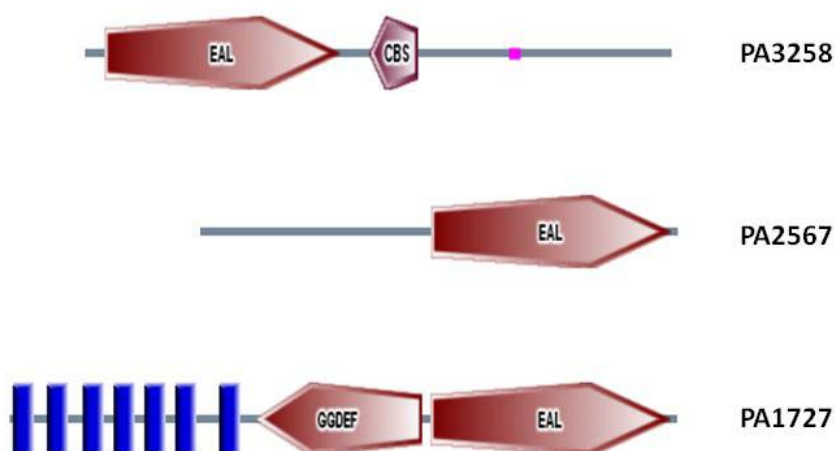
Figure (9) Quantification of biofilm formation in the PA14 strain (A) and the PAO1 strain background (B).

The *P. aeruginosa phoB* mutants exhibited an increased biofilm production (p value < 0.001, t-test), as compared to the control strains (PA14 wild-type and the PAO1 *pcaH* mutant). The *pcaH* mutant was chosen as the PAO1 control strain, because the PAO1 wild-type exhibited an altered resistance-, swimming- and biofilm-expression profile. Complementation of the *phoB* mutant with the *phoBR* genes *in trans* restored the control biofilm phenotype. Biofilm formation was quantified after 24 h of bacterial growth at 37 °C in DeMoss minimal medium (under 0.8 mM and 4 mM P_i , respectively). Mean and standard error were calculated from at least 6 independent replicas.

3.2 Bioinformatic-based search for candidate Pho regulated biofilm genes

Since PhoB in *P. fluorescens* has previously been demonstrated to regulate transcription of a phosphodiesterase, referred to as RapA, which is involved in the degradation of c-di-GMP (Monds *et al.*, 2007), we wondered whether similarly the Pho regulon in *P. aeruginosa* influences transcription of genes encoding for c-di-GMP degrading enzymes, that may be involved in the repression of biofilm formation.

For the identification of down-stream effectors of Pho regulon-mediated biofilm inhibition, we took advantage of the previously published bioinformatic search of the *P. aeruginosa* PAO1 genome for putative Pho boxes (Jensen *et al.*, 2006). Among the 363 putative Pho boxes with a conserved consensus sequence, we found three Pho boxes that were preceding genes predicted to be involved in the modulation of c-di-GMP levels. PA3258, PA2567 and PA1727 encode for proteins with an N-terminal EAL and a C-terminal GGDEF domain (Fig. 10). The Pho boxes and the distances of these boxes from the ATG start codon are listed in Table 3.



<http://smart.embl-heidelberg.de>

Figure (10) Domain organisation of the PA3258, PA2567 and PA1727 proteins.

PA3258 protein has an EAL domain (putative phosphodiesterase activity that degrades c-diGMP) and a CBS domain (domain in cystathionine beta-synthase and other proteins). PA2567 protein harbors only one domain, EAL whereas the protein PA1727 has an EAL domain and a GGDEF domain (putative diguanylate cyclase activity that synthesizes c-di-GMP) as well as 7 repeated transmembrane domains.

3. RESULTS

Table (3) Putative Pho boxes and the distance from the start codon in the promoter region of the genes PA3258, PA2567 and PA1727.

Gene ID	Pho Boxes	Distance from start codon
PA3258	GTCCCATGACTGTTTTAT	- 5
PA2567	ATGTCATTGTGCCTTTAT	-91
PA1727	TTGATATCAACCTGATAT	-36

Of these three genes, PA1727 has previously been shown to encode for a protein that exhibits diguanylate cyclase activity, involved in the production of c-di-GMP, and to contribute to an enhanced biofilm formation (Kulasakara *et al.*, 2006). More recently, it was also shown to enhance alginate production in a mucoid *P. aeruginosa* strain (Hay *et al.*, 2009). PA2567 was shown to exhibit phosphodiesterase activity involved in the degradation of c-di-GMP (Ryan *et al.*, 2006), whereas no activity could be assigned so far for PA3258. However, PA3258 is a putative orthologue of RapA in *P. fluorescens* (76 % aminoacids identity in PA14 and 75 % in PAO1), and was already shown to be involved in c-di-GMP degradation (Monds *et al.*, 2007).

3.3 PA3258 is a member of Pho regulon but not PA1727 or PA2567

To explore whether PA3258, PA2567 and PA1727 were regulated via PhoB in *P. aeruginosa*, we performed semi-quantitative RT-PCR and compared the expression levels of the PA3258, PA2567 and PA1727 transcripts present in the PA14 wild-type under high and low phosphate conditions, respectively. We used the house keeping gene PA1580, encoding for the citrate synthase, as a Pho regulon independent control. Whereas we did not detect any differential in PA1727 and PA2567 gene expression (the latter was only marginally expressed) after 24 h of growth, higher levels of the PA3258 transcript were detected under low phosphate conditions relative to growth under high phosphate conditions in the PA14 wild-type strain, whereas PA3258 transcription was reduced in the *phoB* mutant and enhanced in the *pstS* mutant (Fig. 11).

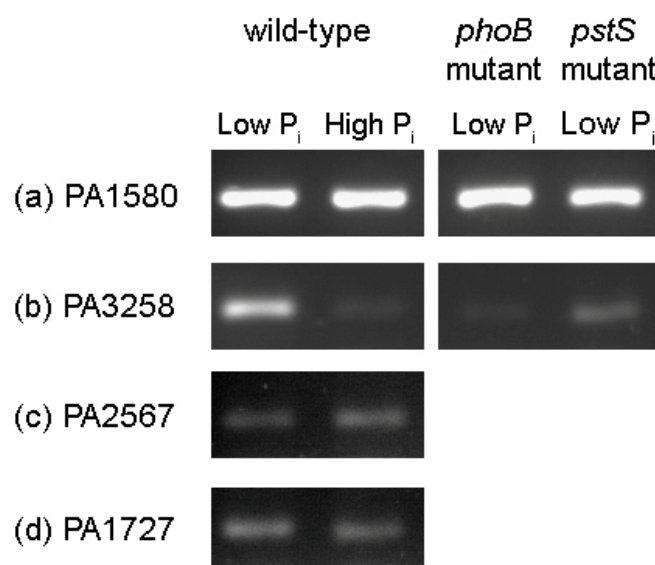


Figure (11) Semi-quantitative RT-PCR for transcription of PA1580 (a), PA3258 (b), PA2567 (c) and PA1727 (d) in *P. aeruginosa* PA14 wild-type under low and high phosphate and in the *phoB* and the *pstS* mutant under low phosphate medium conditions.

The PCR reactions were performed using 10 ng of each cDNA. There was no chromosomal DNA contamination, since no PCR products could be detected, when the RNA was not transcribed into cDNA (data not shown).

Furthermore, we analyzed the transcription of PA3258, PA2567 and PA1727 in the PA14 wild-type under low versus high phosphate conditions over time (Table 4) by performing a real-time quantitative RT-PCR. Only PA3258 exhibited an enhanced transcription at low phosphate medium conditions relative to high phosphate medium conditions, which was maximal at 24h of incubation. These results clearly suggest that PA3258 gene expression is regulated via PhoB in a phosphate-dependent manner.

3. RESULTS

Table (4) Real-time quantitative RT-PCR for transcription of the genes PA3258, PA2567 and PA1727 in the *P. aeruginosa* strain PA14.

The transcription was measured in the wild-type, which was grown under low versus high phosphate growth conditions and samples were taken over time (16h, 24h and 40h of incubation). Mean and standard errors were calculated from 4 independent RNA samples for each time point.

	Transcription fold of RNA under low phosphate relative to high phosphate conditions over time		
	16 h	24 h	40 h
PA3258	1.48 (+/- 0.49)	2.40 (+/- 0.90)	0.78 (+/- 0.42)
PA2567	0.56 (+/- 0.15)	0.52 (+/- 0.35)	0.60 (+/- 0.07)
PA1727	0.93 (+/- 0.58)	0.74 (+/- 0.36)	0.63 (+/- 0.17)

3.4 PA3258 contributes to the inhibition of biofilm formation

In order to evaluate the biofilm phenotype of *P. aeruginosa* mutants that lacked a functional PA3258, PA2567 and PA1727 gene, we tested the respective transposon mutants from the PA14 (PA14_21870, PA14_31330 and PA14_42220) and the PAO1 mutant library, for their capability to form biofilms.

Whereas the PA14 PA2567 mutant did not exhibit an altered biofilm forming capability as compared to the control, loss of the PA14 PA3258 gene entailed an enhanced biofilm formation. However, the PA14 PA3258 exhibited less biofilm as compared to that of the PA14 *phoB* mutant. Complementation of the PA14 PA3258 and the PA14 *phoB* mutants with the PA14 PA3258 gene *in trans* could restore the control biofilm phenotype (Fig. 12). Similarly, as observed before in the PA14 *phoB* mutant (Fig. 9 A), biofilm formation in the PA14 PA3258 mutant was enhanced even under high phosphate medium condition as compared to the PA14 control. The overall biofilm formation in PA14 strain was not significantly influenced by phosphate availability (Fig. 12 A).

In the PAO1 strain, the PA2567 and PA1727 mutants exhibited comparable biofilm forming capability to the control. The PA3258 and PAO1 *phoB* mutants exhibited an enhanced biofilm formation as compared to the control only under low phosphate

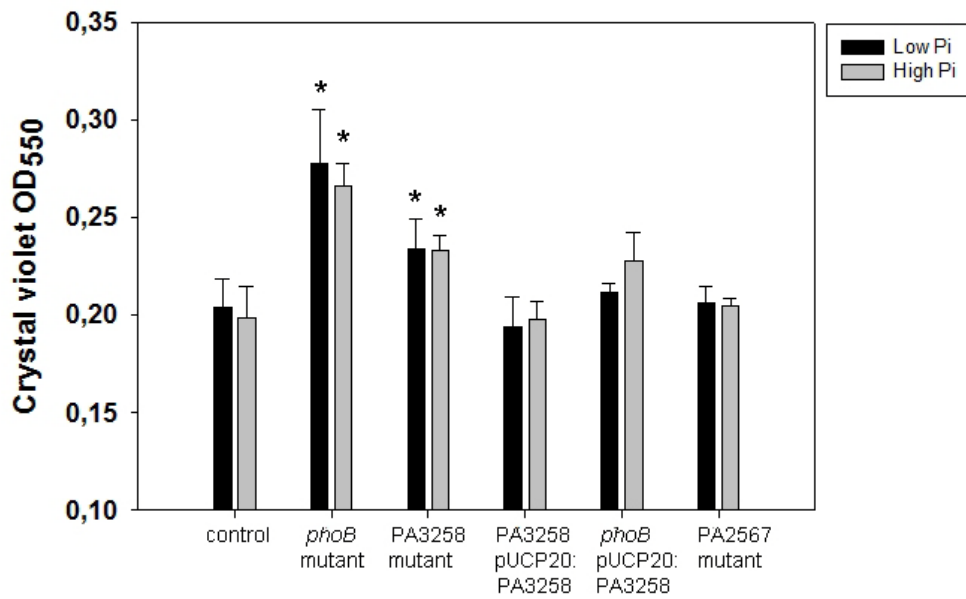
3. RESULTS

conditions (Fig. 12). Complementation of the PA3258 mutant with the respective gene *in trans* restored the control biofilm phenotype. In contrast to PA14 strain, complementation of the PAO1 *phoB* mutant with the PA3258 gene *in trans* couldn't restore the control biofilm phenotype, but conversely, entailed a more enhanced biofilm formation under low phosphate conditions (Fig. 12 B). As shown before in the PAO1 strain, high phosphate significantly enhanced biofilm formation as compared to low phosphate.

These results suggest that PA3258 is involved at least partially in the Pho regulon-dependent inhibition of biofilm formation in *P. aeruginosa*. Since PA3258 is an orthologue of RapA in *P. fluorescens*, we referred to PA3258 as RapA for regulator of adherence by the Pho regulon.

3. RESULTS

A



B

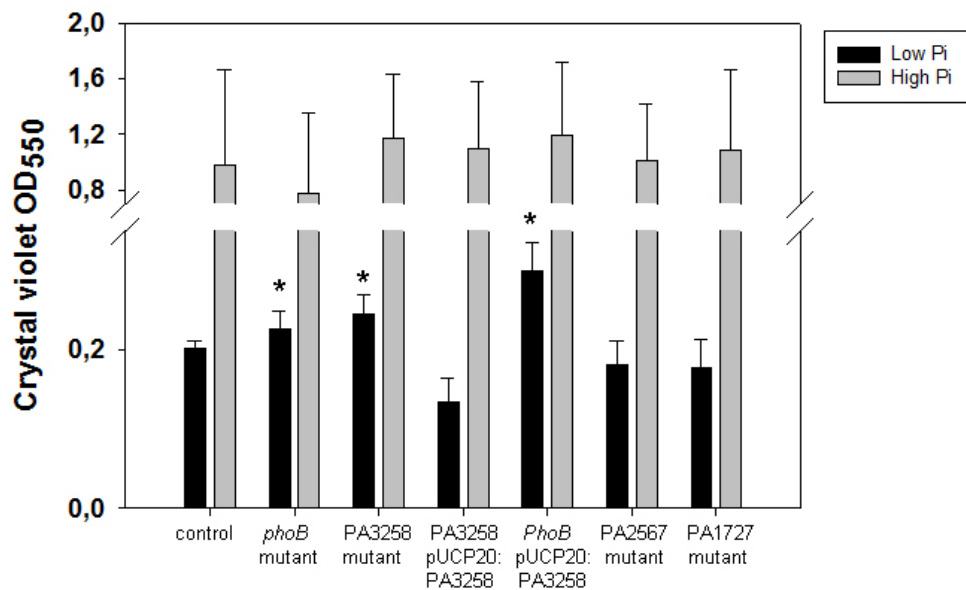


Figure (12) Quantification of biofilm formation in the PA14 strain (A) and the PAO1 strain background (B).

Whereas the PA2567 mutants produced biofilms comparable to that of the PA14 wild-type control, the *rapA* (PA3258) mutants and the *phoB* mutants exhibited an increased production of biofilms ($p < 0.05$, t-test). Complementation of the *rapA* (PA3258) mutant with the *rapA* (PA3258) gene *in trans* (pUCP20:PA3258) restored the control biofilm phenotype. Complementation of the *phoB* mutant with *rapA* (PA3258) gene *in trans* (pUCP20:PA3258) partially restored the wild-type phenotype in PA14 background while the same complementation strongly enhanced the biofilm formation in PAO1 background ($p < 0.01$, t-test). Mean and standard error were calculated from at least 6 independent replicas.

3.5 Pho regulon influences the biofilm structure of *P. aeruginosa*

To investigate the influence of the Pho regulon on the biofilm structure of *P. aeruginosa*, we analyzed the biofilm architecture of the *P. aeruginosa* PA14 wild-type, *phoB* mutant, *rapA* mutant, *pstS* mutant, *phoB* mutant complemented with pUCP20:*phoBR* and *rapA* mutant complemented with pUCP20:*rapA* with a Confocal Laser Scanning Microscope (CLSM). Therefore, we scanned biofilms formed at the bottom of the 96 well plates, in which the bacteria were grown for 72 h. As demonstrated in Fig. (13), similar to the crystal violet staining biofilm assay (Fig. 12 A), the *phoB* mutant exhibited an enhanced biofilm structure and biomass compared to the wild-type, while complementation of the *phoB* mutant with pUCP20:*phoBR* could restore the wild-type level of biofilm structure. The *rapA* mutant showed a slightly enhanced biofilm structure as compared to wild-type and complementation of the *rapA* mutant with pUCP20:*rapA* even reduced the biofilm structure and the biomass. Interestingly, the *pstS* mutant had a flat biofilm structure with only a few scattered micro-colonies.

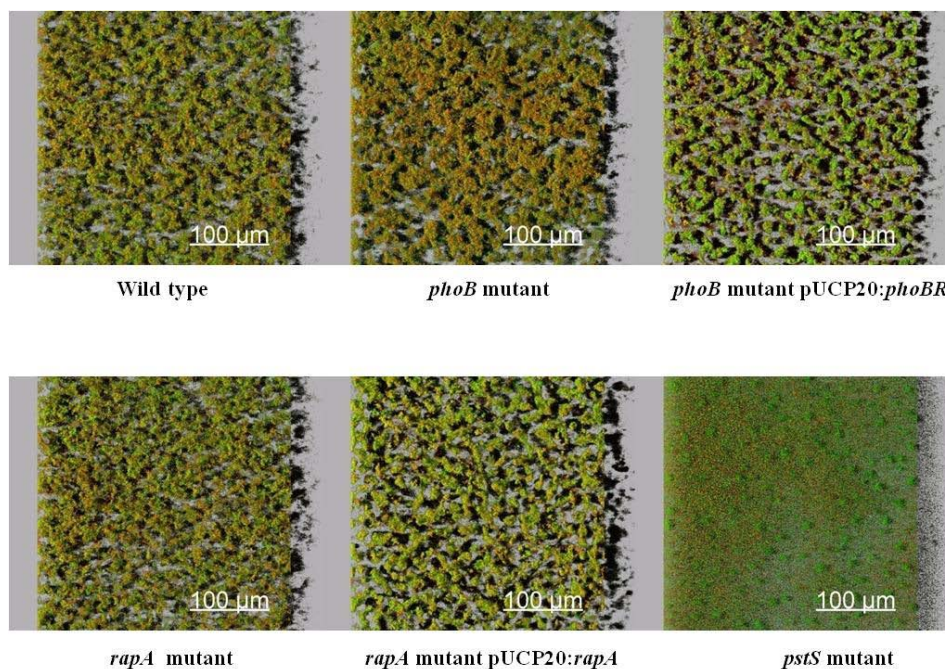


Figure (13) Biofilm structures (3D visualization) of PA14 wild-type, *phoB* mutant, *phoB* mutant complemented with pUCP20:*phoBR*, *rapA* mutant, *rapA* mutant complemented with pUCP20:*rapA* and *pstS* mutant.

Images were acquired from 72 h old biofilms stained with LIVE/DEAD stain using an Olympus Fluoview FV1000 CLSM (lens: UPLSAPO 20x/0.75). The green color represents the living cells, whereas the red color represents the dead cells.

3.6 Expression of CupA fimbriae do not contribute to the Pho-dependent biofilm phenotype

It has been published that CupA fimbriae are important for autoaggregation and biofilm formation of small colony variants of *P. aeruginosa* clinical isolate SCV20265. Moreover, expression of these CupA fimbriae was shown to be modulated by c-di-GMP (Meissner *et al.*, 2007). Thus, we examined the expression of the CupA protein in *P. aeruginosa* PA14 wild-type, *phoB* and *rapA* mutants under low and high P_i concentrations using Western blot analysis. As demonstrated in Fig. (14), there were no differences in CupA fimbriae expression among the tested bacteria. This means that the CupA fimbriae do not involved in the Pho-dependent biofilm regulation in *P. aeruginosa* PA14 strain.

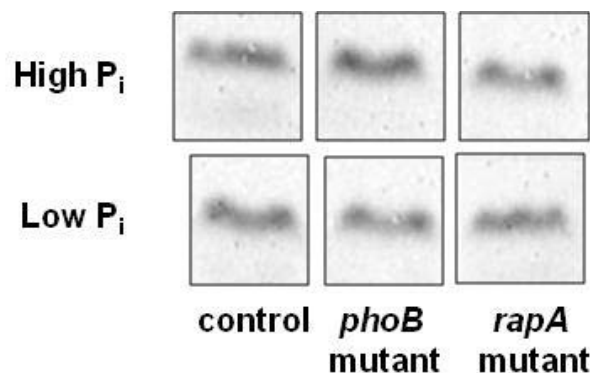


Figure (14) Western blot of CupA fimbriae expression in *P. aeruginosa*.

The tested bacteria (wild-type control, *phoB* and *rapA* mutants) were grown for 24 h in DeMoss minimal medium under low P_i conditions. Western blots were developed with a polyclonal serum directed against the structural subunit of *P. aeruginosa* fimbriae encoded by *cupA1*. The figure is a representative of 3 independent experiments.

3.7 The Psl polysaccharide does not contribute to the Pho-dependent biofilm phenotype

It has been shown that the polysaccharides encoded by *pel* and *psl* operons are important for the biofilm formation in *P. aeruginosa* (Friedman and Kolter, 2004a, b; Matsukawa and Greenberg, 2004). Recently, these polysaccharides were demonstrated to be influenced by the intracellular level of cyclic-di-GMP (Lee *et al.*, 2007). We used Congo red binding assay to examine the influence of Pho regulon on the Psl polysaccharide. As demonstrated in Fig. (15), there were no significant differences in the residual Congo red stain among the tested mutants, i.e. there is no

3. RESULTS

difference in the Psl polysaccharide among the tested strains, which means that the Psl polysaccharide is not involved in the Pho-dependent biofilm phenotype.

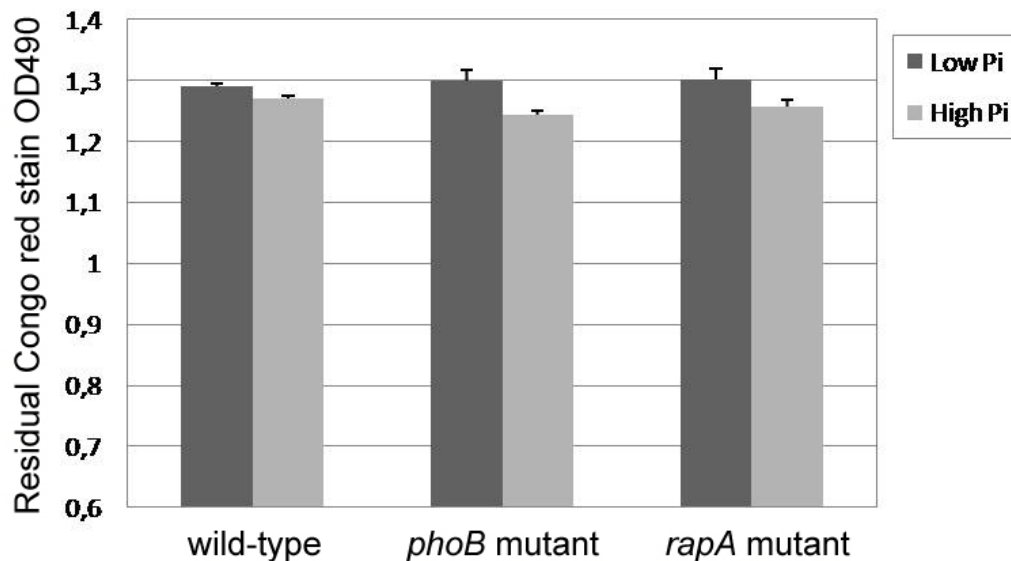


Figure (15) Congo red binding assay of *P. aeruginosa* PA14 *phoB*, *rapA* mutants and wild-type control.

Bacteria were grown for 24 h in DeMoss minimal medium under low or high phosphate conditions and further incubated for 2 h with 40 µg/ml Congo red. The residual Congo red was measured at 490 nm (the amount of Congo red dye that did not bind to the bacteria; lower numbers indicate more Congo red bound to the cells). There was no significant difference in the residual Congo red among the different mutants. Mean and standard error were calculated from 3 independent experiments.

3.8 Expression and purification of the RapA protein to assess the phosphodiesterase activity of RapA *in vitro*

In this work, we identified the RapA protein as a downstream effector of PhoB. RapA is predicted to have an EAL domain. In order to proof phosphodiesterase activity of the RapA protein *in vitro*, we aimed at expressing and purifying the protein. We cloned the *rapA* gene into the expression vector pET21a(+) and could express the RapA protein in *E. coli* BL21(DE3). As shown in Fig. (16), although the RapA protein could be expressed in *E. coli* BL21(DE3) after induction with 0.1 mM IPTG, we couldn't purify RapA as it was within the insoluble fraction.

3. RESULTS

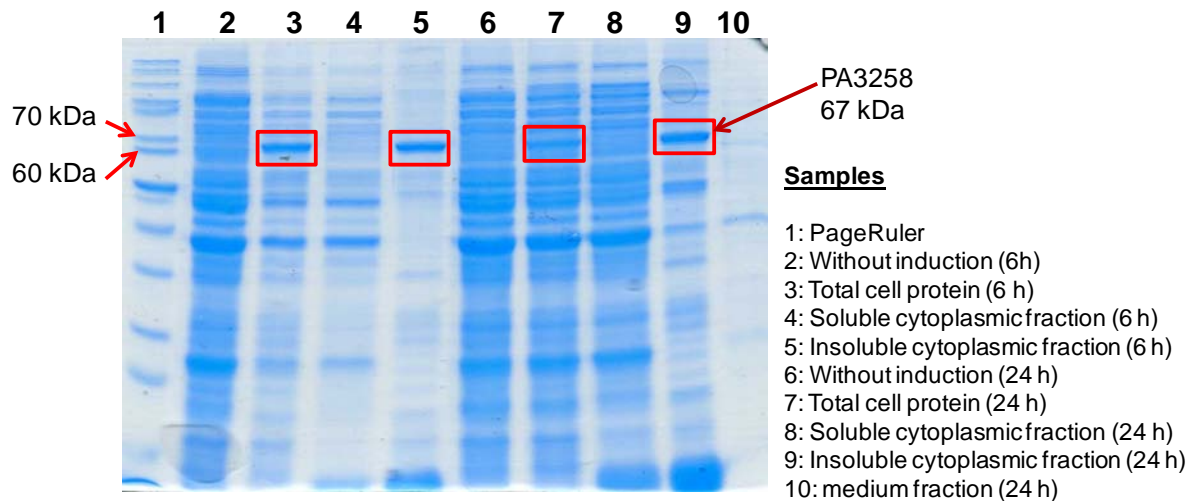


Figure (16) Analytical SDS-PAGE showing the overexpression of the RapA protein in *E. coli* BL21 pET-21a(+)*rapA*.

The SDS-PAGE was running at 120 V for 2 h. The RapA protein was expressed after induction by 0.1 mM IPTG but it was found to be insoluble. The figure is a representative to 3 trials of purification of the RapA protein, which was expressed at different cultivation temperatures (20 °C, 30 °C and 37 °C).

3.9 The Pho regulon influences the type three secretion system

In *P. aeruginosa* c-di-GMP signaling has been demonstrated to be involved in biofilm formation, as well as in the expression of the type three secretion system (TTSS) (Kulasakara *et al.*, 2006), as a major virulence trait. This prompted us to further examine, whether the Pho regulon also affects the expression of the TTSS. We therefore introduced pExoT-gfp (Hornef *et al.*, 2000) into the *P. aeruginosa* PA14 wild-type, the *phoB* mutant, the *rapA* mutant, the *pstS* mutant, and as a control into the *exsA* and the *exsD* mutant, respectively, and determined the intensity of fluorescence of ExoT-GFP-producing bacteria. ExsA is a positive regulator of the TTSS, whereas ExsD negatively regulates the expression of the TTSS.

As depicted in Fig. (17), the *exsA* mutant exhibited an abolished and the *exsD* mutant an enhanced expression of ExoT. We found a clearly enhanced expression of ExoT in the *phoB* mutant as compared to the PA14 wild-type. Significant lower expression of the TTSS was observed in the *pstS* mutant, clearly indicating that the Pho regulon represses ExoT expression in PA14. An enhanced production of the TTSS was also apparent in the *rapA* mutant, albeit to a lower extent as compared to the *phoB* mutant. Expression of ExoT-GFP in PA2567 mutant (not regulated by PhoB) was

3. RESULTS

comparable to the wild-type control. As expected, the *pstS* mutant, where PhoB is constitutively active, expressed less ExoT as compared to the *phoB* mutant or the wild-type control (Fig. 17), again indicating that an active Pho regulon represses the TTSS expression in *P. aeruginosa* PA14. The enhanced TTSS expression was found to be PhoB-dependent but not phosphate-dependent as there was no significant difference of the ExoT expression between the high and low phosphate conditions. These results imply that PhoB is sufficient to suppress TTSS expression even under high phosphate conditions in *P. aeruginosa* PA14. Under these conditions PhoB might be expressed in a basal level or might even be activated via environmental signals other than low phosphate. We also introduced the pExoT-gfp plasmid into the PAO1 mutants; however, no significant fluorescence could be detected in the PAO1 strain background.

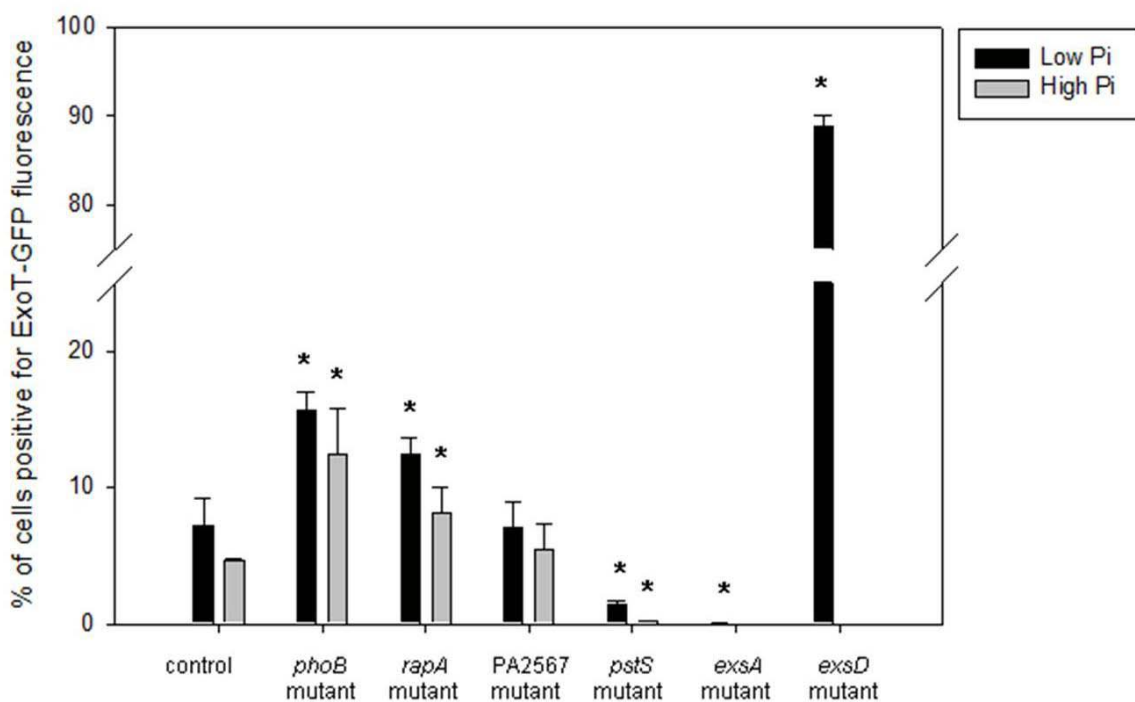


Figure (17) pExoT-gfp expression in *P. aeruginosa* PA14 as measured by FACS analysis.

Expression of ExoT-GFP was clearly enhanced in the *phoB* mutant ($p < 0.01$, t-test) and - albeit to a lower and extend - in the *rapA* mutant ($p < 0.01$, t-test) as compared to the wild-type under low and high phosphate medium conditions. Expression of ExoT-GFP in PA2567 was comparable to the wild-type control. The consecutive activation of the Pho regulon in a *pstS* mutant significantly reduced expression of ExoT-GFP ($p < 0.001$, t-test). The *exsA* and the *exsD* mutants were used as negative and positive controls respectively. Mean and standard error were calculated from 3 independent replicas.

3.10 A PA14 *flgF* mutant negatively regulates biofilm formation similar to the Pho regulon

In order to unravel which factors are important for the establishment and maintenance of *P. aeruginosa* biofilms, a biofilm screening system was established in our group and 5500 transposon mutants of the PA14 mutant library were tested for altered biofilm phenotypes (Musken *et al.*, 2010). In this screen, the *flgF* mutant was found to produce an enhanced biofilm and was used as a positive control for biofilm formation. The *flgF* gene encodes for the flageller basal body rod protein FlgF and belongs to the operon *flgFGHIJKL* that is crucial for the flagella biosynthesis. Although the *flgF* mutant exhibited markedly reduced biofilm formation on abiotic surfaces such as glass tubes (Fig. 18 A), it produced improved biofilm structures and enhanced biomass compared to the wild-type at the bottom of the 96 well plates as estimated by CLSM (Fig. 18 B).

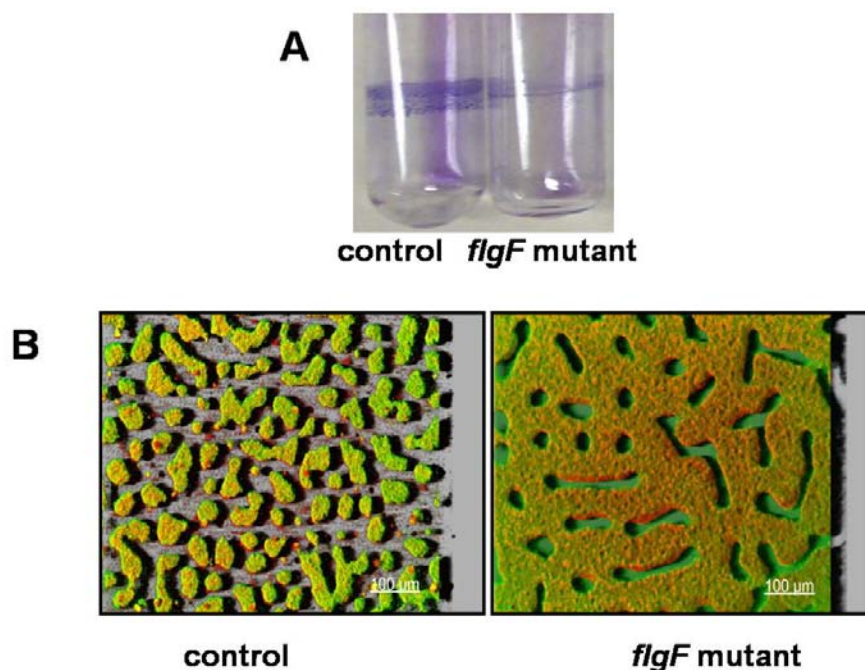


Figure (18) Biofilm formation of the *P. aeruginosa* PA14 wild-type and *flgF* mutant.

(A) The bacteria attached to the walls of the glass tubes were stained with 0.1 % (w/v) Crystal violet after 48 h of growth. The *flgF* mutant showed a reduced attached biofilm compared to the wild-type. **(B)** Biofilms were grown at the bottom of 96 well plates. Images were acquired from 72 h old biofilms stained with LIVE/DEAD stain using an Olympus Fluoview FV1000 Confocal Laser Scanning Microscope (lens: UPLSAPO 20x/0.75). The green color represents the living cells where the red color represents the dead cells.

3.11 TTSS expression is negatively influenced by the FlgF protein

Recently, it has been demonstrated by Soscia *et al.*, 2007 that in a *P. aeruginosa* strain that lacks flagella, TTSS gene expression, effector secretion and cytotoxicity were increased. Since the *flgF* mutant is lacking one crucial gene of the flagella biosynthesis operon and is deficient in swimming motility (Fig. 19), we determined TTSS expression by introducing pExoT-gfp (Hornef *et al.*, 2000) into the *P. aeruginosa* PA14 wild-type, *flgF* mutant and the complemented mutant with the plasmid pUCP20:*flgF* and measured the intensity of fluorescence of ExoT-GFP-producing bacteria by FACS analysis (Fig. 20). As expected, ExoT-GFP expression was clearly enhanced in the *flgF* mutant compared to the wild-type and could be partially restored by complementation of the *flgF* mutant with pUCP20: *flgF*.

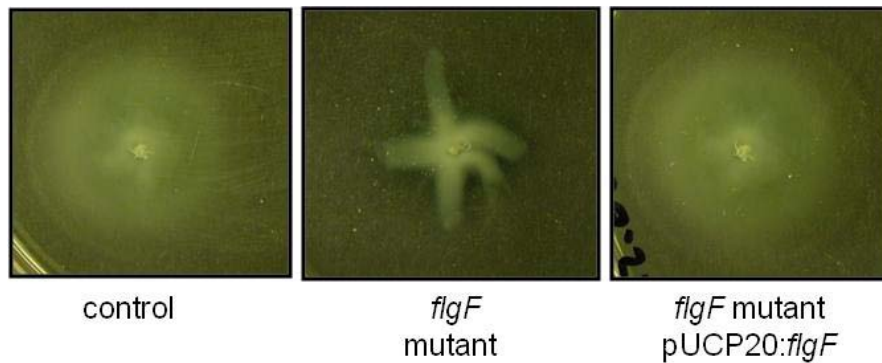


Figure (19) Swimming motility of *P. aeruginosa* PA14 wild-type, *flgF* mutant, *flgF* mutant complemented with pUCP20: *flgF*.

The *flgF* mutant did not swim but it formed a star-shape like zone of swarming on the agar surface around the inoculation point. Swimming motility of the *flgF* mutant could be restored by complementation of the mutant with pUCP20: *flgF*. This figure is a representative of 3 independent experiments.

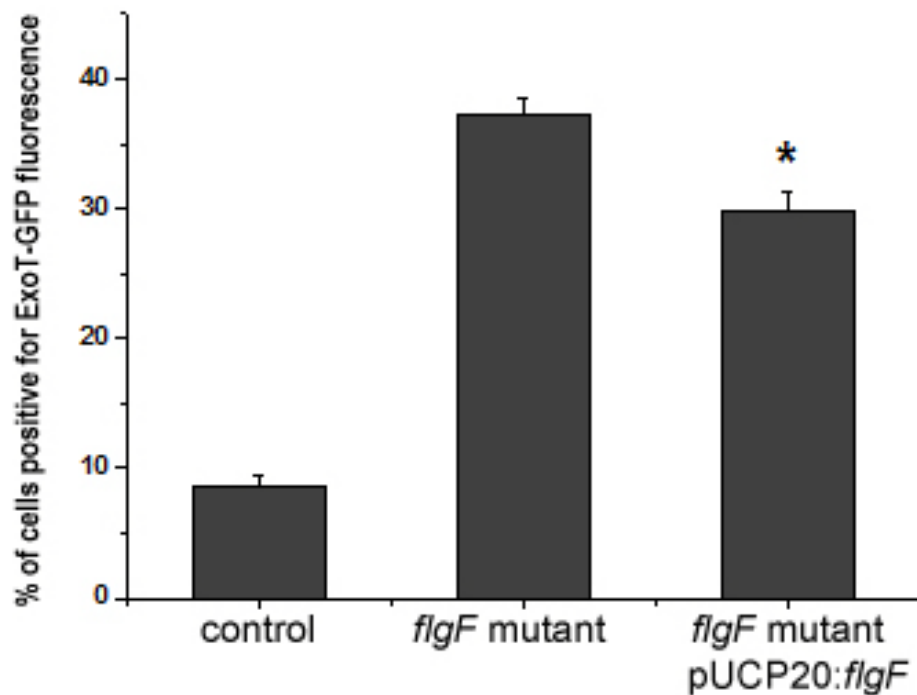


Figure (20) pExoT-gfp expression in the *P. aeruginosa* PA14 wild-type, *flgF* mutant and the mutant complemented with pUCP20:*flgF* as measured by FACS analysis.

Expression of ExoT-GFP was clearly enhanced in the *flgF* mutant ($p < 0.001$, t-test). Complementation of the *flgF* mutant with pUCP20:*flgF* could partially restore the wild-type level of ExoT-GFP expression ($p < 0.01$, t-test). The mean and standard errors were calculated from 3 independent replicas.

3.12 Transcriptome analysis of the *flgF* mutant

Since two bacterial behaviors, biofilm formation and TTSS-mediated virulence are regulated on the one hand by the Pho regulon via - at least partially - RapA and on the other hand by FlgF, we wondered whether the Pho regulon and FlgF might cooperatively affect the *P. aeruginosa* virulence phenotype. Since FlgF affects swimming motility (Fig. 19), while the Pho regulon does not (Fig. 21), we sought to test whether FlgF might control biofilm formation and TTSS via an impact on the Pho regulon.

3. RESULTS

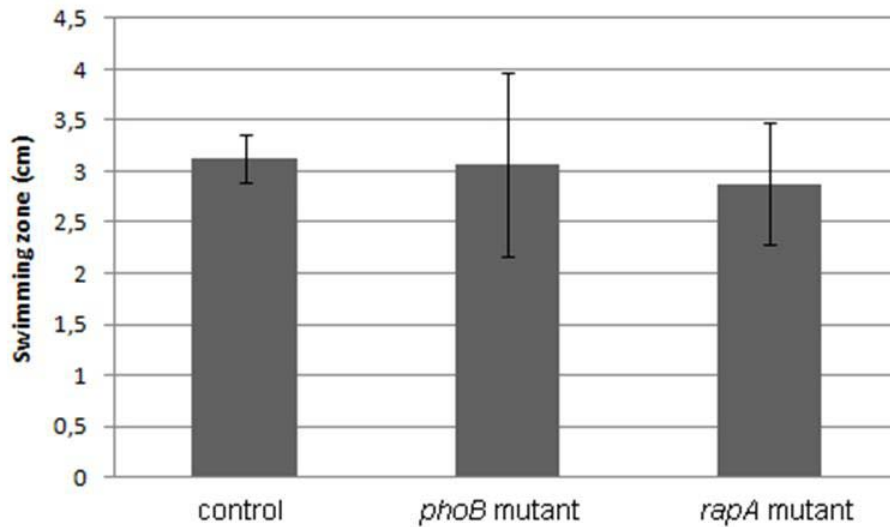


Figure (21) Swimming of *P. aeruginosa* PA14 wild-type control, *phoB* mutant, *rapA* mutant.

Bacteria were inoculated into DeMoss minimal medium low phosphate supplemented with 0.1 % casaminoacid (CAA) and incubated for 24 h at 30 °C. Mean and standard errors were calculated from 3 independent experiments each had 3 independent replicas.

We performed a transcriptome analysis of the PA14 *flgF* mutant. A comparison of the gene expression profile of the *flgF* mutant revealed a total of 130 genes that were differentially expressed at least 2 fold as compared with the control PA14 wild-type culture. Eighty-two of these genes were shown to be up-regulated and forty-eight genes were shown to be repressed in the transcriptome analysis of the PA14 *flgF* mutant.

Although the transcriptome analysis of the *flgF* mutant demonstrated that FlgF is not regulating the PhoB/R regulon, the most remarkable finding of the global transcriptional profile of the *flgF* mutant was that the TTSS encoding genes were strongly up-regulated. Most of genes that are involved in TTSS expression (31 genes), such as those genes encoding for the effector proteins *exoT*, *exoS*, and *exoY*, genes encoding for translocation proteins in TTSS including, *pcsOPQR*, the gene cluster *pcrGVH* encoding for the TTSS regulatory proteins *pcrG* and *pcrH*, the TTSS export proteins encoded by the genes *pscBCDEFGHIJ*, as well as *exsA* encoding for the transcriptional activator of TTSS were up-regulated. A second finding was that the gene *pvdH* involved in pyoverdine synthesis was also up-regulated. Interestingly, genes encoding for proteins required for biosynthesis of pyochelin and its precursors such as *pchABCDERGF* and Fe³⁺-pyochelin outer

3. RESULTS

membrane receptor precursor *ftpA* were strongly down-regulated. The operons *flgBCDE* and *flgFGHIJKL* encoding for flagella biosynthesis were found to be up-regulated. This can be explained as the bacteria were trying to compensate the impaired flagella due to mutation in the *flgF* gene, by up-regulating these biosynthetic operons. Lists of up-regulated and down-regulated genes in the PA14 *flgF* mutant transcriptome profile are provided in Tables 5 and 6, respectively.

Table (5) List of up-regulated genes in the PA14 *flgF* mutant, as compared to PA14 wild-type.

Similarities to other known genes or gene products are provided for genes encoding hypothetical proteins, according to the *Pseudomonas* Genome Database v2 (www.pseudomonas.com).

ORF	Gene	Fold change in regulation	Protein discription
PA0044	<i>exoT</i>	9,207	exoenzyme T
PA0102		2,707	probable carbonic anhydrase
PA0103		4,423	probable sulfate transporter
PA0104		3,305	hypothetical protein
PA1077	<i>flgB</i>	6,197	flagellar basal-body rod protein FlgB
PA1078	<i>flgC</i>	6,154	flagellar basal-body rod protein FlgC
PA1079	<i>flgD</i>	6,656	flagellar basal-body rod modification protein FlgD
PA1080	<i>flgE</i>	6,532	flagellar hook protein FlgE
PA1081	<i>flgF</i>	6,139	flagellar basal-body rod protein FlgF
PA1082	<i>flgG</i>	3,435	flagellar basal-body rod protein FlgG
PA1083	<i>flgH</i>	2,310	flagellar L-ring protein precursor FlgH
PA1084	<i>flgI</i>	2,587	flagellar P-ring protein precursor FlgI
PA1085	<i>flgJ</i>	3,191	flagellar protein FlgJ
PA1086	<i>flgK</i>	4,082	flagellar hook-associated protein 1 FlgK
PA1087	<i>flgL</i>	2,466	flagellar hook-associated protein type 3 FlgL
PA1441		2,491	hypothetical protein
PA1692		3,916	probable translocation protein in type III secretion
PA1693	<i>pscR</i>	2,397	translocation protein in type III secretion
PA1694	<i>pscQ</i>	4,223	translocation protein in type III secretion
PA1695	<i>pscP</i>	3,343	translocation protein in type III secretion
PA1696	<i>pscO</i>	5,206	translocation protein in type III secretion
PA1697		5,646	ATP synthase in type III secretion system
PA1698	<i>popN</i>	2,209	Type III secretion outer membrane protein PopN precursor
PA1699		4,540	conserved hypothetical protein in type III secretion
PA1700		3,321	conserved hypothetical protein in type III secretion
PA1701		5,272	conserved hypothetical protein in type III secretion

3. RESULTS

PA1705	<i>pcrG</i>	5,133	regulator in type III secretion
PA1706	<i>pcrV</i>	11,837	type III secretion protein PcrV
PA1707	<i>pcrH</i>	13,909	regulatory protein PcrH
PA1708	<i>popB</i>	7,731	translocator protein PopB
PA1709	<i>popD</i>	10,600	Translocator outer membrane protein PopD precursor
PA1710	<i>exsC</i>	6,580	exoenzyme S synthesis protein C precursor
PA1711		6,752	hypothetical protein
PA1712	<i>exsB</i>	7,594	exoenzyme S synthesis protein B
PA1713	<i>exsA</i>	6,678	transcriptional regulator ExsA
PA1714		8,427	hypothetical protein
PA1715	<i>pscB</i>	6,501	type III export apparatus protein
PA1716	<i>pscC</i>	3,128	Type III secretion outer membrane protein PscC precursor
PA1717	<i>pscD</i>	3,116	type III export protein PscD
PA1718	<i>pscE</i>	13,332	type III export protein PscE
PA1719	<i>pscF</i>	8,047	type III export protein PscF
PA1720	<i>pscG</i>	7,038	type III export protein PscG
PA1721	<i>pscH</i>	4,603	type III export protein PscH
PA1722	<i>pscI</i>	7,052	type III export protein PscI
PA1723	<i>pscJ</i>	4,564	type III export protein PscJ
PA1966		2,684	hypothetical protein
PA1967		6,995	hypothetical protein
PA2069		2,027	probable carbamoyl transferase
PA2138		2,005	probable ATP-dependent DNA ligase
PA2142		2,142	probable short-chain dehydrogenase
PA2145		2,325	hypothetical protein
PA2146		2,051	conserved hypothetical protein
PA2147	<i>katE</i>	3,474	catalase HPII
PA2148		2,472	conserved hypothetical protein
PA2152		2,235	probable trehalose synthase
PA2153	<i>glgB</i>	3,534	1,4- α -glucan branching enzyme
PA2156		2,167	conserved hypothetical protein
PA2160		4,182	probable glycosyl hydrolase
PA2161		4,554	hypothetical protein
PA2162		3,406	probable glycosyl hydrolase
PA2172		2,038	hypothetical protein
PA2174		2,135	hypothetical protein
PA2191	<i>exoY</i>	6,315	adenylate cyclase ExoY
PA2412		2,496	conserved hypothetical protein
PA2413	<i>pvdH</i>	2,224	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH
PA2693		2,045	conserved hypothetical protein

3. RESULTS

PA2750	2,186	hypothetical protein
PA3094	2,443	tRNA-Val /// tRNA-Asp /// probable transcriptional regulator
PA3711	2,095	probable transcriptional regulator
PA3783	2,129	hypothetical protein
PA3842	7,904	probable chaperone
PA4638	2,220	hypothetical protein
PA5437	3,216	probable transcriptional regulator
PA5532	2,169	hypothetical protein
PA5534	2,646	hypothetical protein
PA5535	3,396	conserved hypothetical protein
PA5536	3,946	conserved hypothetical protein
PA5537	2,329	hypothetical protein
PA5538 <i>amiA</i>	2,760	N-acetylmuramoyl-L-alanine amidase
PA5539	2,204	hypothetical protein
PA5540	2,685	hypothetical protein
PA5541 <i>pyrQ</i>	2,237	dihydroorotase

Table (6) List of down-regulated genes in the PA14 *flgF* mutant, as compared to PA14 wild-type.

Similarities to other known genes or gene products are provided for genes encoding hypothetical proteins, according to the *Pseudomonas* Genome Database v2 (www.pseudomonas.com).

ORF	Gene	Fold change in regulatin	Protein description
PA0179		2,188	probable two-component response regulator
	<i>hcpA</i> /// <i>hcpB</i>		
PA0263	/// <i>hcpC</i>	5,742	secreted protein Hcp /// tRNA-Arg
PA0284		3,239	hypothetical protein
PA0320		5,411	conserved hypothetical protein
PA0505		2,123	hypothetical protein
PA0621		2,165	conserved hypothetical protein
PA0623		2,004	probable bacteriophage protein
PA0627		2,389	conserved hypothetical protein
PA0632		2,074	hypothetical protein
PA0633		2,344	hypothetical protein
PA0634		2,119	hypothetical protein
PA0637		2,010	conserved hypothetical protein
PA0807		2,004	conserved hypothetical protein
PA0887	<i>acsA</i>	2,420	acetyl-coenzyme A synthetase
PA1178	<i>oprH</i>	2,202	PhoP/Q and low Mg ²⁺ inducible outer membrane

3. RESULTS

PA1183	<i>dctA</i>	4,271	protein H1 precursor
PA1561	<i>aer</i>	2,151	C4-dicarboxylate transport protein
PA1656		2,292	aerotaxis receptor Aer
PA1658t		3,094	hypothetical protein
PA2204		2,026	conserved hypothetical protein
PA2331		2,156	probable binding protein component of ABC transporter
PA3038		2,158	hypothetical protein
PA3353		2,339	probable porin
PA3487	<i>pldA</i>	2,027	hypothetical protein
PA3662		7,387	phospholipase D
PA3922		2,327	hypothetical protein
PA3923		2,001	conserved hypothetical protein
PA4131		2,006	hypothetical protein
PA4133		2,509	probable iron-sulfur protein
PA4218		5,721	cytochrome c oxidase subunit (cbb3-type)
PA4219		2,534	probable transporter
PA4220		7,175	hypothetical protein
PA4221	<i>fptA</i>	19,041	hypothetical protein
PA4222		5,876	Fe(III)-pyochelin outer membrane receptor precursor
PA4223		4,249	probable ATP-binding component of ABC transporter
PA4224	<i>pchG</i>	11,488	probable ATP-binding component of ABC transporter
PA4225	<i>pchF</i>	6,193	pyochelin biosynthetic protein PchG
PA4226	<i>pchE</i>	9,939	pyochelin synthetase
PA4227	<i>pchR</i>	3,076	dihydroaeruginosic acid synthetase
PA4228	<i>pchD</i>	17,382	transcriptional regulator PchR
PA4229	<i>pchC</i>	15,228	pyochelin biosynthesis protein PchD
PA4230	<i>pchB</i>	42,493	pyochelin biosynthetic protein PchC
PA4231	<i>pchA</i>	8,739	salicylate biosynthesis protein PchB
PA4306		3,140	salicylate biosynthesis isochorismate synthase
PA4324		2,479	hypothetical protein
PA4413	<i>ftsW</i>	2,300	hypothetical protein
PA4929		2,248	cell division protein FtsW
PA5167		4,536	hypothetical protein
			probable c4-dicarboxylate-binding protein

3.13 The Pho regulon influences pyochelin and pyoverdine production

Since FlgF controls pyochelin and pyoverdine as shown in the transcriptome analysis and both FlgF and the PhoB/R regulon control common phenotypes (inhibition of TTSS expression and biofilm formation), we wondered whether the Pho regulon might also have an influence on pyochelin and pyoverdine production. Pyochelin and pyoverdine production were reduced in the *phoB* mutant compared with the wild-type under low phosphate. Under high phosphate medium conditions pyochelin production was increased in the *phoB* mutant whereas pyoverdine production was comparable to the wild-type (Fig. 22 and 23). Thus taken together, FlgF and PhoB/R regulon control biofilm formation, TTSS, as well as production of pyochelin and pyoverdine.

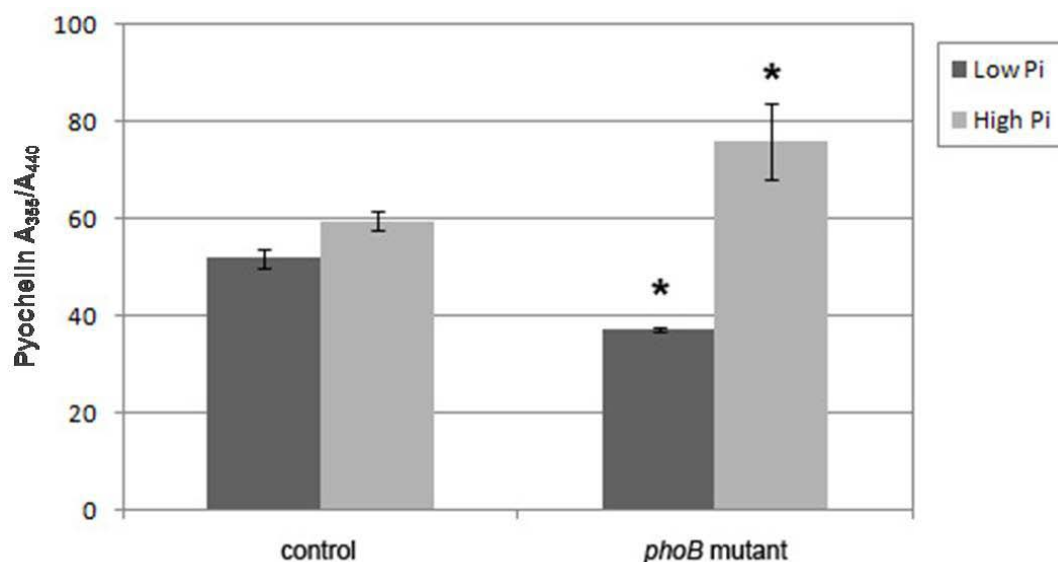


Figure (22) Pyochelin production by *P. aeruginosa* PA14 wild-type control and the *phoB* mutant under low and high phosphate conditions.

Bacteria were grown for 24 h at 37 °C in DeMoss medium under low or high phosphate conditions. Pyochelin production was significantly reduced in the *phoB* mutant compared with the wild-type under low phosphate (P value < 0.0002, t-test) while increased under high phosphate medium conditions (P value < 0.03, t-test). Mean and standard errors were calculated from 3 independent replicas.

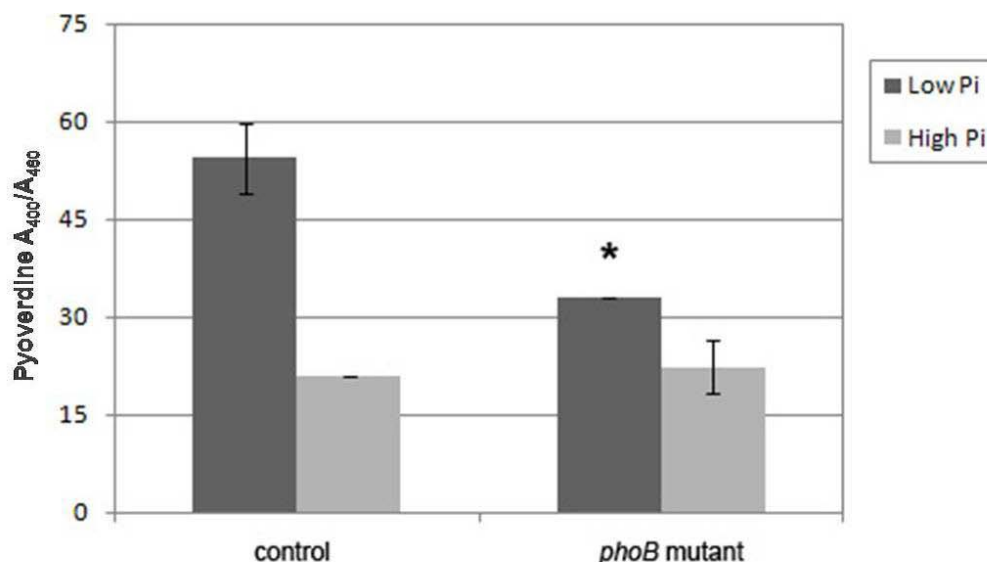


Figure (23) Pyoverdine production by *P. aeruginosa* PA14 wild-type control and the *phoB* mutant under low and high phosphate conditions.

Bacteria were grown for 24 h 37 °C in DeMoss medium under low or high phosphate conditions. Pyoverdine production was significantly reduced in *phoB* mutant compared with the wild-type under low phosphate ($P < 0.003$, t-test). While, there were no difference in the pyoverdine production under high phosphate conditions. Mean and standard errors were calculated from 3 independent replicas.

3.14 Pyochelin and pyoverdine do not affect swimming motility or TTSS

Since the transcriptome analysis of the *flgF* mutant revealed differential regulation of pyoverdine and pyocheline biosynthetic genes, we wondered whether pyoverdine and/or pyocheline influence TTSS directly or through inhibition of flagella expression. We tested the swimming motility and TTSS expression of the *P. aeruginosa* PAO1 wild-type, $\Delta pvdD$, $\Delta pchE/F$ and the double knock out $\Delta pvdD/pchE/F$. As demonstrated in Figure (24), the swimming motility of the pyoverdine, pyocheline and the double knock out mutants were comparable to that of the wild-type. We also tested the expression of ExoT-GFP in the wild-type, $\Delta pvdD$, $\Delta pchE/F$ and the double knock out $\Delta pvdD/pchE/F$ of *P. aeruginosa* PAO1 strain (Fig. 25). There was no significant difference in ExoT-GFP expression among the wild-type and the tested mutants. These results revealed that neither pyocheline nor pyoverdine affect the flagella or TTSS.

3. RESULTS

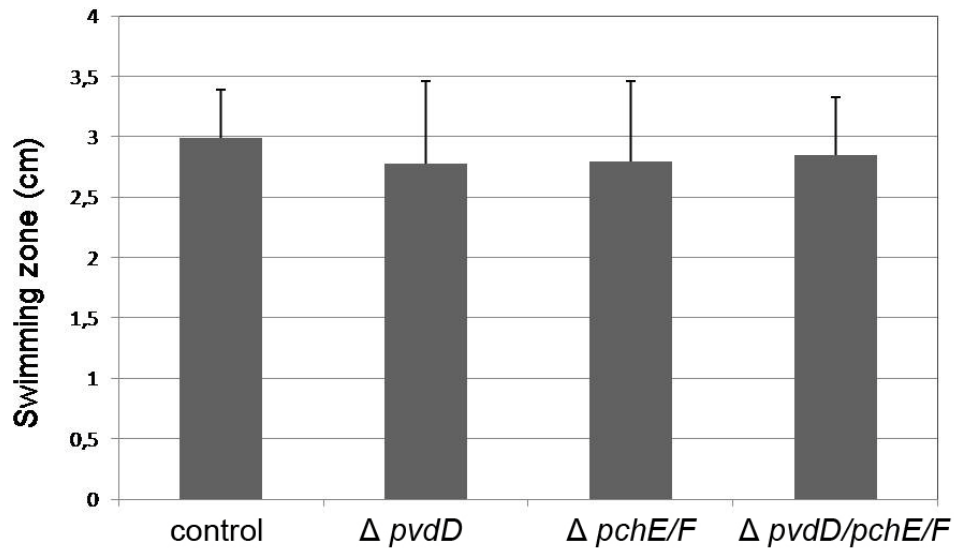


Figure (24) Swimming motility of *P. aeruginosa* PAO1 wild-type, $\Delta pvdD$, $\Delta pchE/F$ and the double knock out $\Delta pvdD/pchE/F$.

The swimming motility of these mutants is comparable to that of the wild-type control and there is no significant difference. Mean and standard errors were calculated from at least 3 independent replicas.

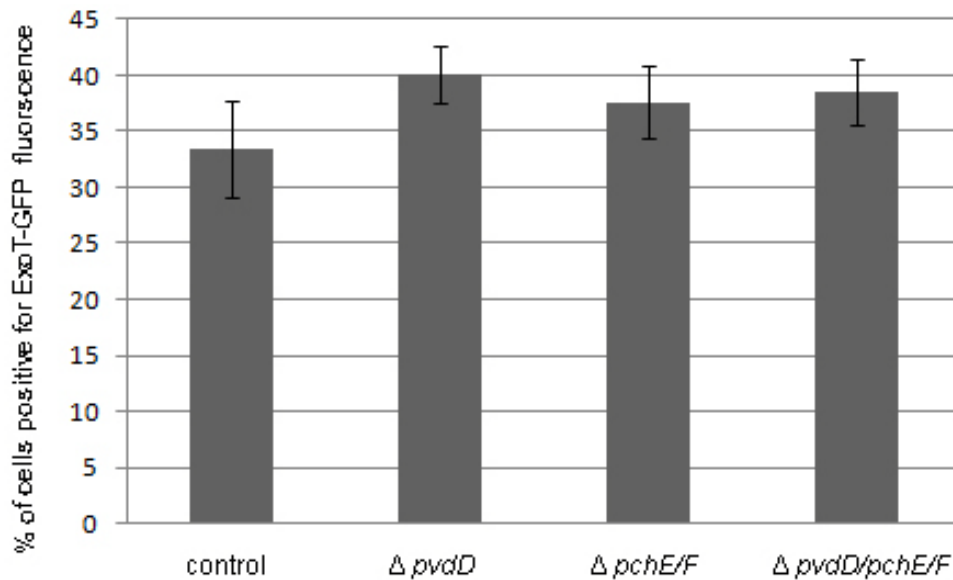


Figure (25) pExoT-gfp expression in the *P. aeruginosa* PAO1 wild-type, $\Delta pvdD$, $\Delta pchE/F$ and the double knock out $\Delta pvdD/pchE/F$ as measured by FACS analysis.

Bacteria were grown in TSB medium under low phosphate conditions for 24 h at 37 °C. there was no significant difference in the ExoT-GFP expression among the wild-type and the tested mutants. Mean and standard errors were calculated from 3 independent replicas.

4 DISCUSSION

P. aeruginosa is the most common Gram-negative bacterium involved in nosocomial infections of immunodeficient patients (Bodey *et al.*, 1983). Acute *P. aeruginosa* infections such as ventilator-associated pneumonia and urinary tract infections are characterized by rapid bacterial growth, eventually followed by sepsis, and can be life-threatening if untreated (Parrillo *et al.*, 1990). Furthermore, *P. aeruginosa* has an exclusive role in the establishment of chronic infections. It is the most common pathogen in chronic cystic fibrosis (CF) lung infections and a major cause of morbidity and mortality in CF patients (Govan and Deretic, 1996; O'Toole and Kolter, 1998; Tummeler and Kiewitz, 1999; Lyczak *et al.*, 2000). Despite the extensive research on *P. aeruginosa* pathogenicity, knowledge about the molecular mechanisms underlying the colonization and establishment of a resistant and persistent biofilm infection remains limited. The ability of *P. aeruginosa* to thrive in many diverse ecological niches as well as to survive in the human host has been attributed to the fine-tuned modulation of gene expression in response to various environmental conditions (Stover *et al.*, 2000; Schuster and Greenberg, 2006; Williams and Camara, 2009). The aim of this work was to study the impact of phosphate as an environmental signal on biofilm formation and virulence of *P. aeruginosa*.

4.1 The Pho regulon influences biofilm formation and TTSS

In this study we present evidence that the Pho regulon inhibits biofilm formation and is required for the repression of the TTSS in *P. aeruginosa* and suggest that this effect is at least in part mediated via a modulation of the intracellular c-di-GMP level. We identified an EAL domain protein RapA as a down-stream effector of the Pho-mediated inhibition.

RapA was shown to be involved in the inhibition of biofilm formation in *P. aeruginosa* PA14 strain (Fig. 12 A and 13) and PAO1 background (Fig. 12 B), as well as in the inhibition of the expression of the TTSS (Fig. 17). In the same context, c-di-GMP signaling has been demonstrated to be involved in biofilm formation, as well as in the expression of the TTSS as a major virulent trait (Kulasakara *et al.*, 2006). Expression analysis of *rapA* in *P. aeruginosa* strain PA14 demonstrated that this gene is a member of the Pho regulon because *rapA* transcription was dependent on the presence of the *phoB* and was enhanced under low phosphate conditions (Fig. 11 and table 4).

Interestingly, in contrast to *P. fluorescens* and *P. aeruginosa* PAO1 the PA14 Pho regulon-dependent inhibition of biofilm formation and repression of TTSS couldn't be reversed by growing PA14 wild-type under high phosphate medium conditions (Fig. 9, 12 and 17). Under these conditions PhoB shouldn't be phosphorylated by PhoR and thus high phosphate conditions might have been expected to mimic a Pho regulon mutant phenotype in the wild-type. We suggest as an explanation for the observed phenotypic differential in the *phoB* mutant as compared to the wild-type even under high phosphate conditions, that the expression of a basal level of PhoB is sufficient to suppress biofilm formation in *P. aeruginosa* PA14 under high phosphate conditions. One might even argue that PhoB in PA14 could be phosphorylated by other means than phosphate, as it has been described before in *E. coli* where PhoB was found to be activated (cross-regulated) by CreC, the sensor kinase of the CreC/CreB two component system which is involved in regulation of carbon catabolism (Wanner and Wilmes-Riesenberg, 1992) or it can also be activated by acetyl phosphate (Kim *et al.*, 1996; Wanner, 1996). This might also explain why biofilm formation in PA14 is significantly lower under high phosphate conditions as opposed to the PAO1 strain and sheds some light onto the molecular mechanisms for strain specific behavior e.g. in the expression of the virulence phenotype.

Although we demonstrated that the Pho regulon is involved in biofilm inhibition at least partially via a c-di-GMP modulating protein, RapA, the Pho regulon does not influence CupA fimbriae or Psl exopolysaccharide, which have been shown to be important for biofilm formation and which are regulated by c-di-GMP in *P. aeruginosa* (Friedman and Kolter, 2004a, b; Matsukawa and Greenberg, 2004; Lee *et al.*, 2007; Meissner *et al.*, 2007). Moreover, we tried to overexpress the RapA protein in *E. coli* to purify the protein for further analysis of its enzymatic function. Unfortunately, although we were able to express RapA in *E. coli* using the pET system, we couldn't purify the protein as it was in the insoluble fraction.

This work clearly indicates that the Pho regulon is important in *P. aeruginosa* to regulate biofilm formation and TTSS mediated virulence, two bacterial behaviors which significantly affect clinical outcome (Hueck, 1998; Costerton, 2001; Roy-Burman *et al.*, 2001; Hauser *et al.*, 2002; Abe *et al.*, 2005). Previous studies indicated an antagonism between the biofilm mode of growth and expression of the TTSS in *P. aeruginosa*. For example, it has been shown that in the RetS-deficient *P.*

aeruginosa strain, the expression of genes encoding for the TTSS were strongly reduced as indicated by transcriptome analysis, while this mutant had an increased ability to form biofilms on both an abiotic surface (glass) and a biotic surface (Goodman *et al.*, 2004). RetS has been shown to repress *pel* gene expression and biofilm formation while activating the TTSS, whereas LadS activates *pel* gene expression and biofilm formation and represses TTSS expression (Ventre *et al.*, 2006). Moreover, the three component system RocARS (SadARS) has been shown to negatively regulate TTSS expression in biofilm forming bacteria, whereas it regulates the expression of the *cup* loci, which contributes to pellicle formation (Kuchma *et al.*, 2005). In contrast, our results demonstrated that both biofilm formation and TTSS expression seem to be under negative control of the Pho regulon. We proposed a model for PhoB-dependent regulation of biofilm formation and TTSS (Fig. 26).

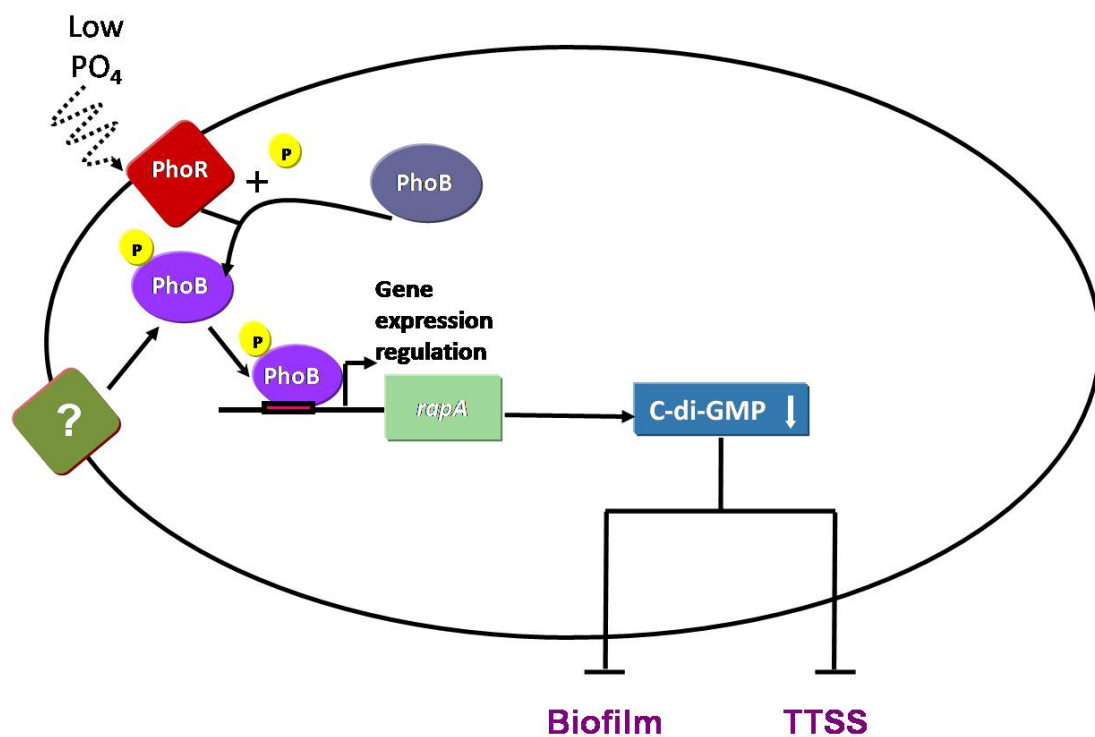


Figure (26) proposed model for PhoB-dependent regulation of the biofilm and virulence phenotype.

Under low P_i conditions, the two component system PhoR sensor kinase autophosphorylates and activates its response regulator PhoB, which binds to the Pho boxes in the promoter region of *rapA* and induces transcription and expression of this gene. The RapA protein has an EAL domain exhibiting PDE activity and reducing the cellular level of c-di-GMP, which in turn inhibits biofilm formation and TTSS. Some other factors or sensors may activate the PhoB independent of the environmental phosphate availability.

4.2 Expression of flagella influences biofilm formation and TTSS

Flagella have been shown to be involved in regulation of biofilm formation and they are important for initial attachment to surfaces (O'Toole and Kolter, 1998). Similar to the Pho regulon, a PA14 *flgF* mutant negatively regulates biofilm formation as this mutant was used as a control for enhanced biofilm formation in a screening of *P. aeruginosa* PA14 mutant library for mutants exhibiting an altered biofilm phenotype (Musken *et al.*, 2010). The *flgF* mutant exhibited an enhanced biofilm structure and biomass compared to the wild-type at the bottom of 96-well plates as observed by confocal laser scanning microscope (Fig 18 B). However, the *flgF* mutant was shown to exhibit a limited biofilm formation on glass tubes surfaces as compared to the wild-type as demonstrated by crystal violet staining of surface attached biofilm (Fig. 18 A). In the same context, whereas a flagella mutant, which was grown in a static culture was shown to exhibit a limited biofilm formation on a PVC surface as compared to wild-type (O'Toole and Kolter, 1998), another flagella mutant, which was grown in a flow chamber was shown to form a thicker and hilly biofilm as compared to the carpet-like biofilm formed by the wild-type (Klausen *et al.*, 2003b). The differences are likely due to the media used because it has been demonstrated that biofilms grown in the presence of glucose (Stewart *et al.*, 1993; Davies *et al.*, 1998) are different from those grown in the presence of citrate (Heydorn *et al.*, 2000; Heydorn *et al.*, 2002).

Recently, flagellar assembly and TTSS expression have been shown to be inversely regulated in *P. aeruginosa* as in a strain lacking flagella, TTSS gene expression, effector secretion, and cytotoxicity were increased. Conversely, flagellar-gene expression and motility were decreased in a strain overproducing ExsA, the TTSS master regulator (Soscia *et al.*, 2007). Similarly, our results indicated that the flagella negatively influence the TTSS. The *flgF* mutant exhibited strongly enhanced ExoT-GFP expression as compared to the wild-type and this enhanced ExoT-GFP expression could be partially restored by complementation of the *flgF* mutant with pUCP20:*flgF* (Fig. 20).

4.3 Transcriptome analysis does not uncover cooperative regulation of biofilm formation and TTSS expression

Our results indicate that a non-motile *flgF* mutant exhibits a similar phenotype as *phoB* mutant in respect to enhanced biofilm formation and TTSS expression. Therefore, we tested whether the FlgF and the PhoB are controlling biofilm formation and TTSS expression in *P. aeruginosa* via similar mechanisms by applying transcriptome analysis. However, the transcriptome analysis of the *flgF* mutant compared to the wild-type did not uncover an influence on the Pho regulon. Moreover, we found that the Pho regulon did not control FlgF or flagella as it did not influence swimming motility (Fig. 21).

The phenotypes controlled by the Pho regulon and the *flgF* mutant are demonstrated in Fig. (27). The Pho regulon activates expression of *rapA* gene. The RapA protein harbors an EAL domain, which exhibits PDE activity and reduces the cellular level of c-di-GMP. The low level of c-di-GMP inhibits both of biofilm and TTSS. FlgF is essential for the expression of the flagella, which is required for inhibition of biofilm formation and repression of TTSS expression.

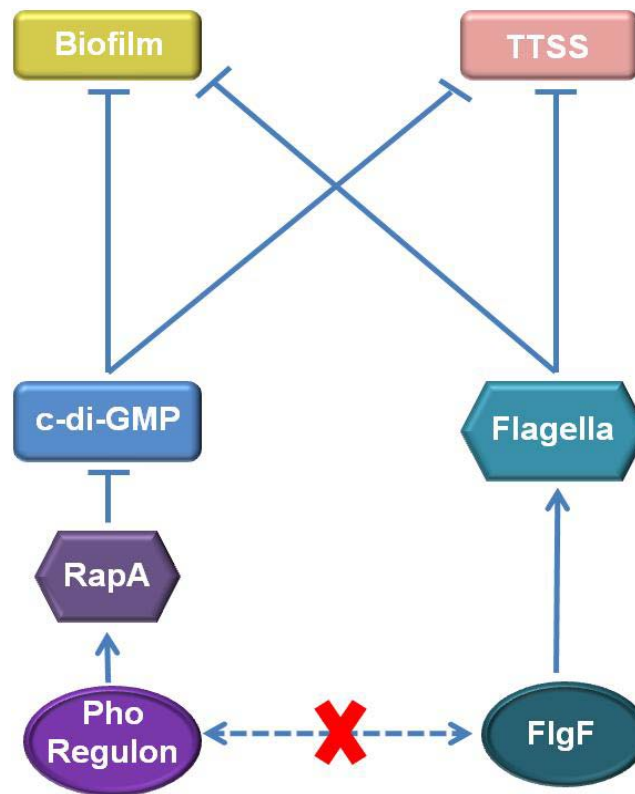


Figure (27) Phenotypes controlled by the Pho regulon and FlgF.

Both the Pho regulon and the flagella negatively influence biofilm formation and TTSS in *P. aeruginosa*. The Pho regulon activates *rapA* expression. The RapA protein harbors an EAL domain, which exhibits PDE activity and reduces the cellular level of c-di-GMP, which in turn reduces the cellular level of c-di-GMP due to its PDE activity. The low level of c-di-GMP inhibits both of biofilm and TTSS. FlgF is essential for the expression of the flagella, which is required for inhibition of biofilm formation and repression of TTSS expression. We found that the Pho regulon did not control FlgF/ flagella as it did not influence swimming motility. Moreover, the transcriptome analysis of the *flgF* mutant compared to the wild-type did not uncover an influence on the Pho regulon.

4.4 PHO regulon and Flagella expression influence pyochelin and pyoverdine production

Pyoverdine and pyochelin are iron chelators which are produced by *P. aeruginosa* under limited iron conditions and are under the negative control of the ferric uptake regulator, Fur, which represses the iron uptake genes (siderophore biosynthesis, receptors) when bound to its co-repressor Fe^{2+} (Escobar *et al.*, 1999). In this study we found that the production of the pyochelin and pyoverdine is influenced by PhoB. The *phoB* mutant produced lower amounts of pyochelin as compared to the wild-type under low phosphate conditions and pyoverdine production was repressed in the *phoB* mutant compared to the wild-type. There was no difference in pyoverdine production under high phosphate (Fig. 22 and 23). This means that these

siderophores are positively regulated by the Pho regulon in an iron-independent way because we did the experiments in a rich iron medium conditions at which, the expression of iron uptake genes was turned off by Fe^{2+} -Fur complex (Crosa, 1997; Escolar *et al.*, 1999).

The transcriptome analysis of the *flgF* mutant indicated clearly that several genes encoding for pyochelin synthesis such as *pchG,F,E,R,D,C,B,A* and the Fe^{3+} -pyochelin outer membrane receptor precursor *FptA* were strongly down-regulated (table 6) while a precursor of pyoverdine, the *pvdH* was found to be up-regulated (table 5). This means that the FlgF, similar to Pho regulon, positively influence pyochelin production but in contrast, negatively influence pyoverdine production and this effect is also iron independent. These results may uncover a new role of pyochelin and/or pyoverdine apart from iron uptake or even add more complexity to regulation of siderophores production in an iron independent way.

To test whether the pyochelin and or pyoverdine are involved in the enhanced expression of TTSS directly or indirectly via repression of the flagella (Soscia *et al.*, 2007), we test swimming motility and TTSS expression of *P. aeruginosa* PAO1 pyochelin and pyoverdine mutants. Swimming motility and TTSS expression of the *P. aeruginosa* PAO1 pyochelin and pyoverdine mutants was comparable to that of the wild-type control (Fig. 24 and 25). These results indicated that pyoverdine and pyochelin do not contribute in the enhanced TTSS-mediated virulence phenotype regulated by Pho regulon and FlgF.

Summary of the important findings of this work are illustrated in Fig. (28).

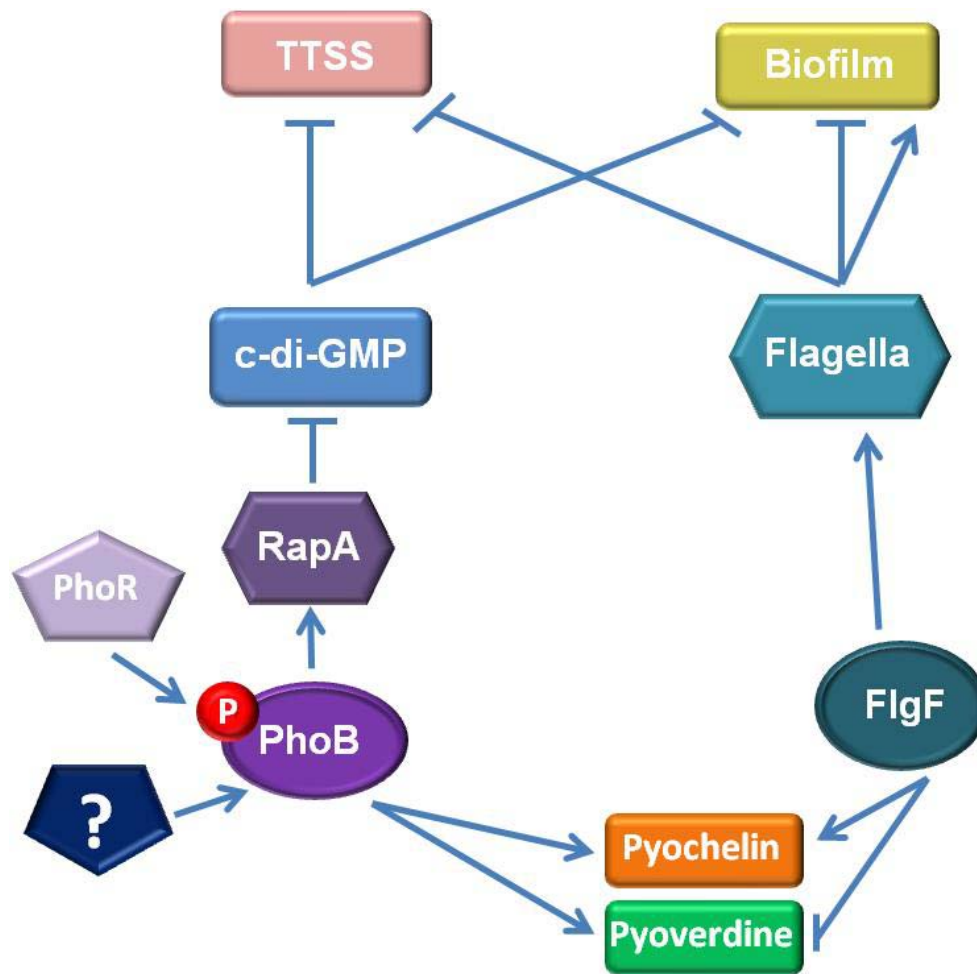


Figure (28) Proposed model for the regulation of biofilm formation and TTSS by the Pho regulon and FlgF.

Under low P_i condition, the sensor kinase PhoR autophosphorylates and activates its partner response regulator PhoB, which binds to the Pho box in the promoter of the *rapA* gene and activates transcription and expression of this gene. The RapA protein harbors an EAL domain, which exhibits PDE activity and reduces the cellular level of c-di-GMP, which in turn inhibits biofilm formation and the expression of TTSS. PhoB may also be activated by other phosphate independent means. Moreover, FlgF - required for flagella synthesis - was found to control biofilm formation and also to inhibit the expression of TTSS. We found that the Pho regulon doesn't influence swimming motility and the Pho regulon was not differentially regulated in the transcriptome analysis of the *flgF* mutant. In addition, pyochelin and pyoverdine were found to be influenced by on one hand, the Pho regulon (production of both siderophores were reduced in the *phoB* mutant as compared to the wild-type), and on the other hand, by FlgF. Several pyochelin encoding genes were strongly down-regulated in the transcriptome of *flgF* mutant, while the pyoverdine encoding gene *pvdH* was up-regulated. Investigation of swimming motility (flagella) and TTSS expression in pyochelin and pyoverdine mutants revealed that there is no significant difference as compared to the wild-type.

4.5 Conclusion

It has previously been demonstrated that the Pho regulon influences interbacterial signaling via the 4-quinolones and homoserine lactones in *P. aeruginosa* and thus impacts on the expression of quorum sensing-dependent virulence factors (Jensen *et al.*, 2006). The finding of this study that the Pho regulon also influences a protein involved in the modulation of the intracellular c-di-GMP level adds another level of complexity to the regulation of bacterial behavior. The *P. aeruginosa* genome encodes for multiple GGDEF/EAL domain proteins and various environmental factors seem to impact on their enzymatic activity. However, since the decision to change the bacterial lifestyle from biofilm formation to the establishment of a highly virulent phenotype is very critical, only a fine-tuned local and spatial regulation of the c-di-GMP concentration within the cell will put the bacterial cell into the position to integrate multiple environmental cues and probably also data on the physiological status to carefully establish a c-di-GMP governed bacterial virulence phenotype. Although much has to be learned about the influence of the Pho regulon on the c-di-GMP level under various environmental conditions, and many more questions arise in the context of the link of quorum sensing-dependent and environmental signal-dependent regulation of virulence and c-di-GMP signaling, this work significantly contributes to the unraveling the complex regulation of biofilm formation and the expression of the TTSS in *P. aeruginosa*.

5 SUMMARY

5. SUMMARY

Research into the importance of phosphate as an environmental signal molecule for multicellular behavior in the Pseudomonads *P. aureofaciens* and *P. fluorescens* recently uncovered a role of the Pho regulon in the formation of bacterial biofilms. Most interestingly, it was clearly demonstrated that PhoB activates a phosphodiesterase which decreases the intracellular c-di-GMP levels in *P. fluorescens*, thus providing a link between the Pho regulon and c-di-GMP signaling pathways that impact on biofilm formation.

In this study we present evidence that in the opportunistic pathogen *P. aeruginosa* the Pho regulon inhibits biofilm formation and is required for the repression of the TTSS. We furthermore identified an EAL domain protein, referred to as RapA, as a down-stream effector of the Pho regulon, which at least partially mediated the observed inhibition. Although the Pho regulon contributed to inhibition of biofilm formation, it had no effect on CupA fimbriae expression or Psl exopolysaccharide expression, the common factors contribute to biofilm formation in *P. aeruginosa*. Interestingly, in contrast to *P. aeruginosa* PAO1 strain, the observed inhibition of the biofilm formation was Pho regulon-dependent but independent of the availability of inorganic phosphate in PA14 strain. The Pho regulon-dependent repression of TTSS was also independent of the availability of inorganic phosphate in PA14 strain. These results add even more complexity to the regulation of the bacterial behavior by environmental cues.

We demonstrated that a wild-type derived *flgF* mutant exhibited an enhanced biofilm formation as compared to the wild-type as shown by CSLM. Moreover, ExoT expression was strongly enhanced in the *flgF* mutant as compared to the wild-type indicating that the FlgF, similar to Pho regulon, negatively influences virulence phenotype (inhibition of biofilm formation and repression of the type three secretion system). We found that Pho regulon has no influence on the FlgF or flagella. Although the transcriptome analysis of the *flgF* mutant compared to the wild-type did not uncover an influence of FlgF on Pho regulon, it confirmed that the TTSS is negative regulated by the FlgF because most of genes encoding for TTSS were up-regulated. Moreover, pyochelin encoding genes were strongly down-regulated. We illustrated that both of the Pho regulon and the FlgF affect the production of siderophores. The pyochelin production was enhanced by PhoB and FlgF. The pyoverdine production was enhanced by PhoB but inhibited by FlgF. We also found

5. SUMMARY

that both of the Pho regulon and the FlgF are regulating the same virulence phenotype but independently. Our results add more complexity to the regulation of biofilm formation and TTSS expression in *P. aeruginosa*.

6 ZUSAMMENFASSUNG

Untersuchungen über die Bedeutung von Phosphat als umweltbedingtes Signalmolekül für ein multizelluläres Verhalten der Pseudomonaden *P. aureofaciens* und *P. fluorescens* haben kürzlich die Rolle des Pho Regulons in der Ausbildung von Biofilmen enthüllt. Interessanterweise wurde eindeutig gezeigt, dass PhoB eine Phosphodiesterase aktiviert, die den intrazellulären c-di-GMP Spiegel in *P. fluorescens* absenkt. Hierdurch wird eine Verknüpfung zwischen dem Pho Regulon und c-di-GMP Signalwegen, welche ebenfalls einen Einfluss auf die Biofilmbildung haben, erstellt.

In dieser Arbeit wurde nachgewiesen, dass das Pho Regulon auch in dem opportunistischen Krankheitserreger *P. aeruginosa* die Biofilmbildung inhibiert und für die Unterdrückung des Typ-Drei-Sekretionssystems benötigt wird. Weiterhin wurde als ein Downstream Effektor des Pho Regulons ein EAL-Domänen Protein identifiziert, welches RapA genannt wurde und zumindest teilweise die beobachtete Inhibierung vermittelte. Obwohl das Pho Regulon zu einer Inhibierung der Biofilmbildung beitrug, hatte es keinen Effekt auf die Expression von CupA Fimbrien oder Psl Polysacchariden - allgemeinen Faktoren die zur Ausbildung von Biofilmen in *P. aeruginosa* beitragen. Im Gegensatz zu dem Stamm PAO1 war die beobachtete Hemmung der Biofilmbildung in dem Stamm PA14 zwar abhängig von dem Pho Regulon, aber unabhängig von Vorkommen an anorganischem Phosphat. Die Pho abhängige Unterdrückung des Typ-Drei-Sekretionssystems in PA14 war ebenfalls unabhängig von Vorkommen an anorganischen Phosphat. Diese Ergebnisse unterstreichen die Komplexität der Regulation bakteriellen Verhaltens durch Einflüsse aus der Umwelt.

Weiterhin konnten wir durch CLSM zeigen, dass eine vom Wildtyp abstammende *flgF* Mutante eine erhöhte Biofilmbildung im Vergleich zum Wildtyp aufweist. Außerdem war die ExoT Expression in der *flgF* Mutante im Vergleich zum Wildtyp stark erhöht. Das deutet darauf hin, dass FlgF, ähnlich wie das Pho-Regulon, den Virulenz-Phänotyp negativ beeinflusst (Inhibierung der Biofilmbildung und Unterdrückung des Typ-Drei-Sekretionssystems). Nachforschungen haben ergeben, dass das Pho Regulon keinen Einfluss auf FlgF oder Flagellen besitzt. Eine Transkriptionsanalyse der *flgF* Mutante im Vergleich zum Wildtyp ließ ebenfalls keinen Einfluss von FlgF auf das Pho-Regulon erkennen. Es wurde jedoch die negative Regulation des Typ-Drei-Sekretionssystems durch FlgF bestätigt, da die meisten Gene, die für das Typ-Drei-

Sekretionssystem kodieren, in der *flgF* Mutante hochreguliert waren. Zusätzlich waren Pyochelin kodierende Gene stark herunterreguliert. Schließlich konnten wir zeigen, dass beides, das Pho Regulon und FlgF, die Produktion von Siderophoren beeinflusst. Während die Pyochelinsynthese durch PhoB und FlgF erhöht wurde, wurde die Produktion von Pyoverdin durch PhoB erhöht, durch FlgF jedoch gehemmt. Wir konnten ebenfalls demonstrieren, dass das Pho Regulon und FlgF den selben Virulenz- Phänotyp regulieren, jedoch unabhängig voneinander.

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